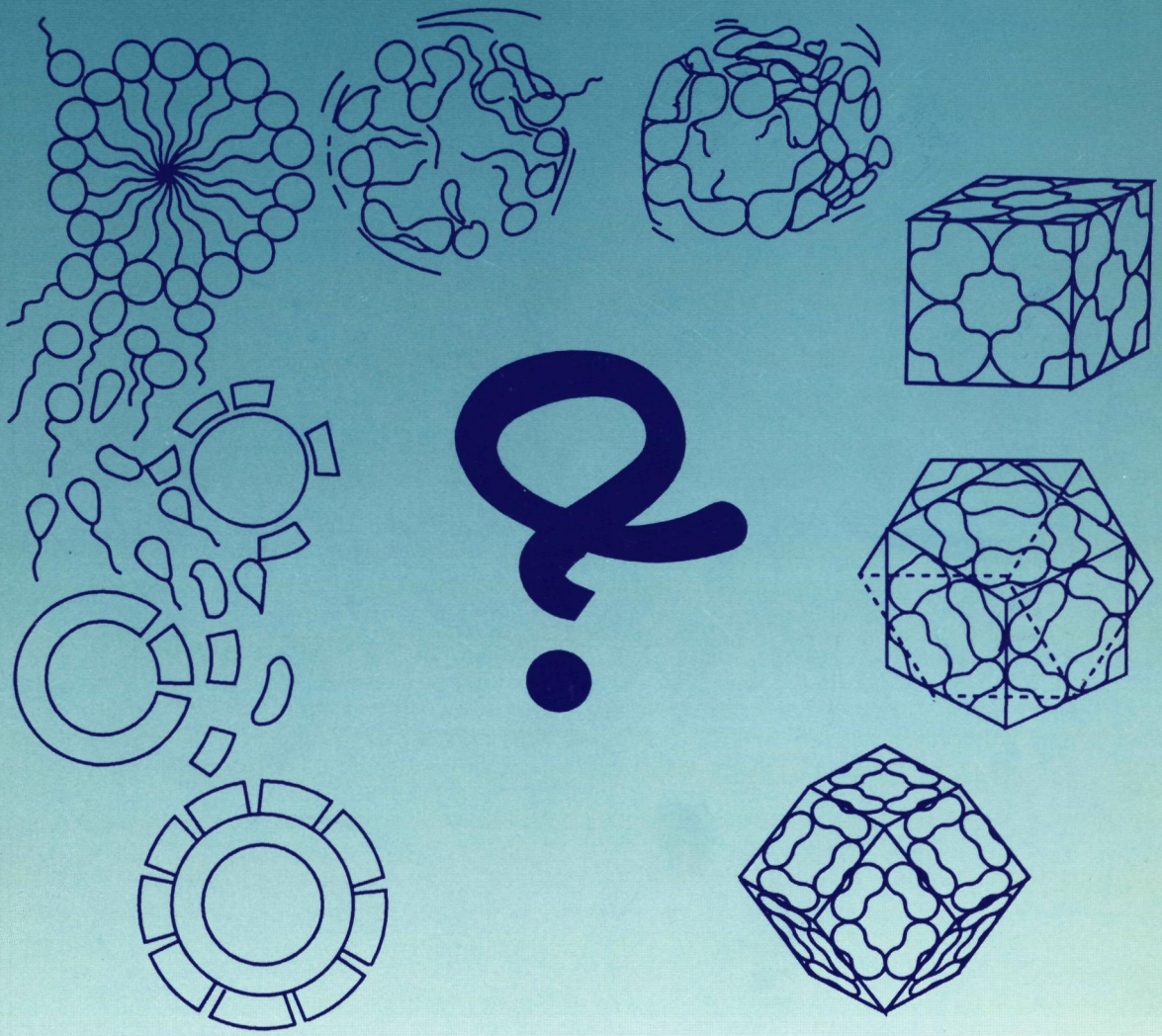
Structural and functional aspects of α-crystallin and its relation to the small heat shock proteins



Karin B. Merck

Structural and functional aspects of α-crystallin and its relation to the small heat shock proteins

Structural and functional aspects of α-crystallin and its relation to the small heat shock proteins

een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen.

Proefschrift

ter verkrijging van de graad van doctor aan de
Katholieke Universiteit Nijmegen,
volgens besluit van het College der Decanen
in het openbaar te verdedigen op
maandag 26 april 1993,
des namiddags te 1.30 uur precies

door
Karin Beatrice Merck
geboren op 28 maart 1964
te Dordrecht

Promotor: **Prof. Dr. H. Bloemendal**

Co-referent: **Prof. Dr. W.W. de Jong**

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Merck, Karin Beatrice

Structural and functional aspects of α-crystallin and its relation to the small heat shock proteins.
/ Karin Beatrice Merck. - [S.l.:s.n.]. - Ill.
Proefschrift Nijmegen. - Met samenvatting in het Nederlands.
ISBN 90-9005952-0
Trefw.: α-crystalline.

The investigations described in this thesis were carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) and performed under the direction of Prof. Dr. W.W. de Jong and Prof. Dr. H. Bloemendal at the Department of Biochemistry, Faculty of Science, University of Nijmegen, Nijmegen, The Netherlands.
This thesis was supported by the 'Merck'sche Gesellschaft für Kunst und Wissenschaft' and Filtron Technology B.V.

ISBN: 90-9005952-0

DANKWOORD

Het schrijven van een dankwoord is per definitie ondankbaar werk. Het is niet mogelijk om iedereen die ik zou willen bedanken te noemen en op één pagina te verwoorden wat de diverse mensen voor mij betekend hebben.
Laat ik beginnen met Willeke. Je hebt je niet alleen voor je dochter maar ook voor mij compleet uit de naad gewerkt. Het werk was soms ondankbaar en de uit te voeren opdrachten leken vaak onmogelijk. Echter, hoewel je soms de wanhoop nabij was, heb je altijd met onverminderde energie en met succes doorgewerkt. Bedankt Willeke!
Christien en Wiljan, jullie wil ik bedanken voor het geduld waarmee jullie een onnozel technoloogje hebben ingewijd in de eiwit-chemische en moleculair-biologische geheimen.
Hoewel mijn andere collega's mij voornamelijk gedwarsboomd hebben door het bezetten van apparatuur als ik die net zelf nodig had, zal ik jullie dan toch maar bedanken. Zonder jullie kwebbelende, roddelende en giebelende aanwezigheid zou het vreselijk saai zijn geweest op het lab. Patricia, Perry, Christien, Paul, Ine, Will, Liesbeth, Els, Francy, John, Jack, Gert-Jan, Geert, Ronald, Wiljan en Wilma, bedankt voor jullie bijdrage aan de absoluut niet-wetenschappelijke discussies. Perry, jou wil ik bedanken voor het vlot uitvoeren van de allerlaatste experimenten, die nog een hoop extra's toevoegden aan de laatste hoofdstukken.
Verder ben ik rijkelijk voorzien geweest van hardwerkende en enthousiaste studenten. Antoinette, Eric, Belinda, John, Frank en Monique, bedankt voor jullie inzet.
I would like to thank you, Joe Horwitz for doing many experiments, very quickly and enthousiastically. By discovering the chaperone activity of α-crystallin and by giving me the opportunity to collaborate with you, you really gave an extra dimension to my work.
Naast al die mensen op het lab is er nog een aantal mensen dat mij buiten het lab op de been heeft gehouden. Op de eerste plaats natuurlijk Martin. Ik weet eigenlijk niet of ik nu ondanks of dankzij jou dit boekje nog vol heb kunnen krijgen. Je sleepte me van de bergen naar het triathlonparcours, de schouwburg, etc. etc. Mocht ik ooit het idee gehad hebben tijd te kort te komen om ook nog te promoveren, dan was ik deze 'stressy' gedachte door al die afleidende activiteiten zo weer kwijt.
Annemarie, je hebt het misschien niet in de gaten gehad, maar ook jij hebt een belangrijke bijdrage geleverd aan dit boekje. Door met vaart en verve onze stek op orde te houden heb je ons een hoop werk uit handen genomen en daardoor ons de gelegenheid gegeven na het werk te ontspannen.
Tenslotte bedank ik mijn vader en moeder die me gedwongen hebben elke dag helemaal naar het VWO te fietsen en me de gelegenheid hebben geboden te studeren.

Voor mijn ouders
Voor Martin

CONTENTS

Chapter 1	Introduction	11
Chapter 2	Structural and functional similarities of bovine α-crystallin and mouse small heat shock protein. A family of chaperones.	29
Chapter 3	Expression and aggregation of recombinant αA-crystallin and its two domains.	45
Chapter 4	Comparison of the homologous carboxy-terminal domain and tail of α-crystallin and small heat shock protein.	61
Chapter 5	Exploring the aggregation and chaperone behaviour of αA-crystallin by site-directed mutagenesis.	75
Chapter 6	Characterization of anti-crystallin autoantibodies in patients with cataract.	89
Summary/Samenvatting		99
List of publications		105
Curriculum Vitae		107

CHAPTER 1

Introduction

INTRODUCTION

α-Crystallin is one of the major vertebrate eye lens proteins. It is usually isolated in the form of large water-soluble aggregates with an average molecular mass of 800,000 Da. The aggregate consists of two types of subunits, αA- and αB-crystallin (173 and 175 amino acid residues, respectively). In rodents a product of alternative splicing, αA^{ins}, forms part of the aggregate. The two subunits show 57% sequence homology.

Already in the nineteenth century, Mörner [1] was able to isolate α-crystallin from bovine lenses. Much later, the amino acid sequences of αA and αB of numerous vertebrate species were determined [2-4], and in 1982 it was found that the α-crystallin subunits are homologous with the ubiquitous small heat shock proteins (HSPs) [5]. Until 1989 α-crystallin was considered as a typical lens-specific structural protein. However, in that year, it was discovered by several research groups that αB-crystallin also occurs outside the lens, notably in heart, striated muscle and kidney [6-8]. In 1991, Kato and co-workers were able to detect very small amounts of αA-crystallin in non-lens tissues [9]. Klemenz and co-workers showed in 1991 that αB-crystallin in NIH 3T3 cells behaves in several ways like the small HSPs [10]. In this context it is interesting to realize that a lot of other crystallins, which are very abundant in the lens, are also present, but at much lower concentrations, in many other tissues, where they function as housekeeping enzymes [11-15]. With these findings, the lens-specificity and strictly structural nature of all crystallins is no longer valid. It is now assumed that normal metabolic proteins were recruited as components of the lens fibers during the evolution of the eye, to serve a structural role in the lens, rather than playing their normal metabolic role. Their physical properties seem to allow association into a highly transparent structure.

From the above it follows that in the course of time the character of α-crystallin research, starting with the view that α-crystallin is a lens-structural protein and finally showing that αB-crystallin is a small HSP, has strongly evolved. Some functional aspects of α-crystallin, particularly as an extralenticular protein and its relation to the small HSPs, are summarized in the following paragraphs. Additionally, a more elaborate overview of the research concerning α-crystallin structure is given.

THE FUNCTION OF α-CRYSTALLIN AND ITS RELATION TO THE SMALL HSPS

Since the discoveries that the α-crystallins are structurally related to the small HSPs [4, 5, 16] and that αB-crystallin expression is not restricted to the lens [6-8], a number of papers appeared on the extralenticular role of αB-crystallin in normal and diseased tissues. In the meantime, many reports on the functional aspects of the small HSPs were published, so that gradually more insight in the role of extralenticular α-crystallin and of the small HSPs is gained. In this part of the introduction a short summary will be given on the structural and functional relationship between α-crystallin, especially αB, and the small HSPs. For a more complete

overview of this extensive field of research, the reader is referred to a recent review [17].

α-Crystallin outside the lens

αA- as well as αB-crystallin occurs in tissues outside the lens. The highest levels of αB-crystallin are reached in heart, striated muscle and kidney. Up to 5% of soluble αB-crystallin is found in rat soleus muscle [18]. The highest levels of αA-crystallin -17 ng/mg- are detected in rat spleen [9].

Small heat shock proteins

The small HSPs form one of the four major groups of heat shock or stress proteins. The three larger groups of HSPs (60, 70 and 90 kDa) are known to be molecular chaperones [19, 20]. They are helpful in the folding and translocation of polypeptides, especially in stress situations, when their genes are induced. The function of the small HSPs is less clear, although there is growing evidence that they too are engaged in protein-protein interactions. Several studies have suggested that the small HSPs are responsible for acquired thermotolerance [21-24]. On the other hand, no effect of inactivation of the yeast HSP27 gene on temperature-sensitivity and thermotolerance development could be detected [25, 26]. The small HSPs form a diverse family of proteins (15-30 kDa) but they all tend to aggregate. Different organisms have a different number of small HSPs, ranging from 1 in yeast and chicken [26, 27] to about 30 in higher plants [28].

Structural similarities of the small HSPs and the α-crystallins

The α-crystallins and the small HSPs are members of a gene superfamily [4, 29]. This is based on a conserved homologous sequence of 90-100 amino acid residues. This gene superfamily also includes two mycobacterial surface antigens and a major egg antigen of *Schistosoma mansoni*. The sequence homology is most pronounced in the C-terminal parts of the polypeptides, corresponding with the putative globular C-terminal domain of α-crystallin [30]. α-Crystallin residues which have been suggested to be of structural importance [30] are very well conserved in the entire family. The N-terminal regions vary in length, and sequence homology cannot be detected.

It was recently shown that the secondary structure of calf α-crystallin and mouse HSP25 are very similar, both proteins having primarily β-sheet conformation [31]. Also hydrophobicity similarities of the homologous region of α-crystallin and the small HSPs have often been noticed [4, 16].

Both α-crystallins and the small HSPs form large aggregates. α-Crystallin is mostly isolated as 800 kDa aggregates, but complexes, ranging in size from 280 to 10,000 kDa have been isolated as well [32, 33]. Also small HSP aggregates differ in size, ranging from 180 kDa for chicken [34] to 400-800 kDa for mammalian small HSPs [35]. Upon stress, αB-crystallin in heart forms larger aggregates [36]. Also small HSP aggregates increase in size upon stress in various cell types [e.g. 35, 37]. Often, concomitantly, the small HSPs redistribute from the cytoplasm towards the perinuclear region or into the nucleus [35, 38]. This phenomenon has also been observed with αB-crystallin in mammalian cells [10, 39, 40]. In all cases redistribution back to the cytoplasm occurs during recovery. Mixed aggregates of αB-crystallin and small HSPs have been observed *in vivo* [41, 42]. In the electron microscope both α-crystallin and the small HSPs appear as 10-18 nm globular, sometimes torus-like or hollow-core particles [37, 43-45].

Like for α-crystallin, the arrangement of subunits within the small HSP aggregate remains unclear. Several models for the

quaternary structure of α-crystallin have been proposed (see later in this introduction). For mouse HSP25 a sphere-like structure composed of about 32 monomers, arranged in hexagonal packing, has been proposed [45].

Gene structure, expression and regulation

The αA- and αB-crystallin genes, located on the human chromosome 21 and 11, respectively [46, 47], contain two introns at homologous positions. The first intron coincides precisely with the intron in the small HSPs genes of *Caenorhabditis elegans*. Most other small HSPs are encoded by intronless genes. The 5' flanking regions of the small HSP genes and of the αB-crystallin gene contain one or more heat shock elements. As a consequence these genes can be induced by elevated temperatures and various other types of stress [10, 48, 49]. Additionally, the small HSP and αB-crystallin genes are induced at specific stages during normal development [e.g. 18, 43, 50, 51]. Small HSP genes can also be induced by estrogen [52] and ecdysteroids [48]. Interestingly, αB-crystallin accumulation in NIH 3T3 cells is brought about by dexamethasone-induction of v-mos and the Ha-ras oncogenes [10, 53, 54].

Some functional aspects

A major common feature of α-crystallin and small HSPs is their phosphorylation on specific serine residues. The phosphorylations of the α-crystallin subunits are most likely catalysed by a cAMP-dependent kinase [55, 56]. Phosphorylation in the lens epithelium is thought to be reversible [57]. Also in other tissues αB-crystallin can be phosphorylated, like in mouse heart and in Alexander's disease brain [58]. The reversibility of the phosphorylation of α-crystallin suggests that it is part of some regulatory mechanism. Phosphorylation of small HSPs is more evidently subject to metabolic control. Phosphorylation of mammalian small HSPs occurs in response to a number of mitogenic and environmental stimuli [59-62]. Phosphorylation of human HSP27 occurs after activation of platelets by thrombin [63] and after heat shock [64, 65].

αB-Crystallin appears to have a role in cells that undergo major cytomorphological reorganizations in early chicken embryogenesis [66]. Likewise, HSP27 of *Drosophila* is abundant during embryogenesis [43]. Additionally, both the small HSPs and αB-crystallin are found to be involved in intracellular changes during disease progression [e.g. 67, 68]. αB-Crystallin is involved in reactive processes of astrocytes and oligodendrocytes in the central nervous system [69], in the development of benign tumors associated with tuberous sclerosis [70], in the development of astrocytic tumors [71] and it is a major component of ubiquitinated inclusion bodies in human degenerative diseases [72]. In these inclusions αB-crystallin is tightly associated with intermediate filaments and ubiquitin [73]. Likewise, small HSP expression seems to be differentially regulated in tumor development [74-76]. It thus appears that αB-crystallin as well as the small HSPs are involved in cellular rearrangements during normal development and disease progression.

In this context it should be mentioned that there are many examples of the interaction of α-crystallin and the small HSPs with membranes and other proteins. Native α-crystallin associates specifically with lens membranes [77, 78] and the lens fiber cytoskeleton, notably the intermediate filaments [79, 80]. Additionally, αB-crystallin interacts specifically with actin in heart [36] and associates with desmin in rat myocytes [44]. Plant chloroplast small HSPs become associated with the thylakoid membranes after heat shock [81, 82]. Cytoplasmic plant small HSPs associate with the cytoskeleton during heat

shock [83].
Recent *in vitro* experiments have shown that both α-crystallin and mouse HSP25 can act like molecular chaperones [31, 84, 85]. This and the fact that α-crystallin and the small HSPs are involved in cytomorphological rearrangements and that they interact with membranes and proteins *in vivo*, suggest that α-crystallin and the small HSPs might be molecular chaperones *in vivo*, as well. The recruitment of α-crystallin as a major lens protein now becomes also understandable. Its intrinsic structural stability makes it suitable to reside life-long, without turnover, in the lens. By preventing undesirable protein interactions and refolding unfolded proteins it would contribute to the maintenance of lens transparency and integrity. In fact, the constitutively high level of α-crystallin in the lens might make it permanently stress-tolerant.

THE STRUCTURE OF α-CRYSTALLIN

It is now well known that the occurrence of α-crystallin is not restricted to the lens and that its function is not strictly structural. However, most of the studies of α-crystallin structure were done at the time that α-crystallin was considered to be a lens-specific structural protein. α-Crystallin structural studies were done in the context of lens transparency. To achieve transparency, light-scattering must be reduced to a minimum. Lens membranes are therefore closely packed and regularly spaced. Cell organelles, such as nucleus, mitochondria and endoplasmatic reticulum are lost upon terminal differentiation of the elongating fiber cells. Consequently, there is no significant transcriptional- and translational activity in the deeper layers of the lens. High protein concentrations are needed to generate a refractive index gradient for proper light-focusing. Protein concentrations in the lens are so high (up to 60% of the lens wet weight) that through short-range interprotein interactions a regular ordening in the position of near-neighbour proteins is produced, resulting in a perfectly clear lens with optimal reduction of light-scattering [86].
From the above it is obvious that strict demands are made on lens proteins. They should be stable, highly soluble, and have a structure that results in close packing and regular ordening. To better understand the architecture of the eye lens, it is therefore crucial to know the three-dimensional structure of the crystallins. The first crystallin for which the crystal structure was solved is the γB-crystallin monomer in 1981 [87], followed by γC in 1986 [88], γE in 1989 [89] and the βB2 homodimer in 1990 [90]. All these crystallins, which are members of one β/γ crystallin family, are comprised of two globular domains, each folded in two symmetrically organized Greek key motifs. Also the organization of the β-crystallin oligomers becomes more and more clear. It is known how the subunits in the βB2 homodimer are interacting, and a computer model of the βA4/βB2 heterodimer has also been built [91].
Unfortunately, the tertiary structure of the α-crystallin subunits and the organization of the subunits within the aggregate are poorly understood. Many attempts have been made to crystallize α-crystallin, however no α-crystallin crystals could be obtained up till now, probably because of the polydisperse nature of the aggregate [92]. Not only is the α-crystallin aggregate composed of two types of primary gene products, αA and αB, but also several posttranslationally modified products [e.g. 56, 93-96] form part of the ag-

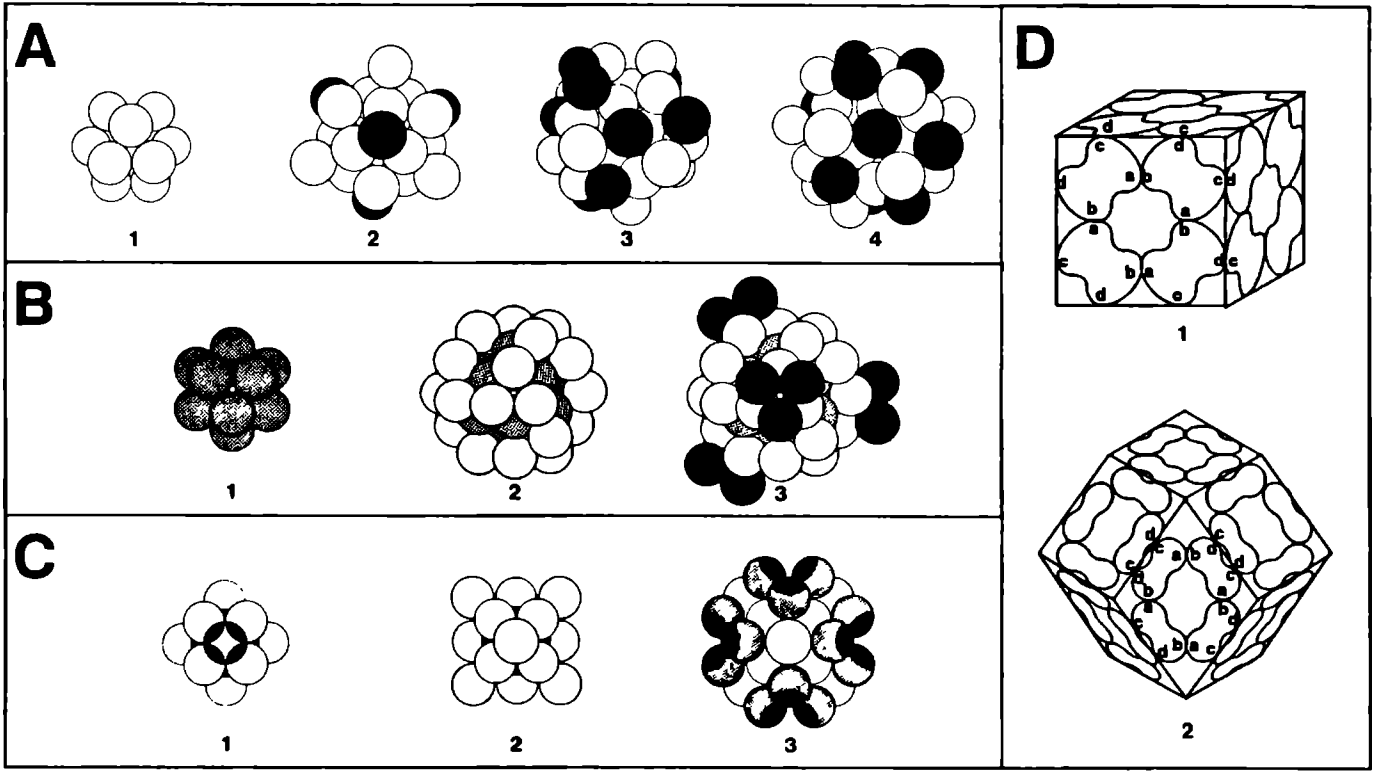
gregate. Structural studies are therefore dealing with a rather complex protein aggregate.

α-Crystallin mainly contains β-pleated sheets and almost no α-helices [97-99], as shown by CD spectroscopy. As stated before, the tertiary structure is not known, but several hypothetical models have been suggested. Some of them are based on the principle that three-dimensional structure similarity can be achieved with very little sequence homology. On basis of hydrophobic residue alignment of α- and β/γ-crystallins, α-crystallin has been proposed to consist of six folding units, arranged in a two- or three-domain structure [100]. Argos and Siezen then compared α-crystallin with the β/γ-crystallins with regard to 'surrounding' hydrophobicity, turn preference and anti-parallel strand potential. This resulted in a four-motif structure for the α-crystallin subunits [101]. This model was endorsed by hydropathy prediction plots [102], far UV CD measurements and secondary structure predictions [97]. Wistow proposed the overall structure of α-crystallin to consist of a globular N-terminal domain of two symmetry-related motifs and a somewhat larger C-terminal domain, also of two motifs, with an exposed C-terminal arm [30]. This model is based on the gene structure and internal homology of αA-crystallin. The N-terminal domain corresponds with exon 1, whereas the entire C-terminal region is encoded by exons 2 and 3. The C-terminal globular domain is common also to the small heat shock proteins and might be the result of duplication of a gene encoding an ancestral 30-40 residue protein. The conserved C-terminal domain probably represents an extremely thermodynamically stable structure which predated the lens, and was used to build a protein capable of surviving for years without turnover in the lens. Indeed α-crystallin is remarkably thermostable [103, 104]. Also the small HSPs are reported to be very stable *in vivo* [61]. Ingolia and Craig, however, postulated that this region of homology between α-crystallin and the small HSPs is an 'aggregation' domain [5], since also the small HSPs form ~800 kDa aggregates.

Also for the quaternary structure of α-crystallin, several models have been proposed. Bindels *et al.* introduced a 'three-layer model' [105]. This model is mainly based on studies that imply three different environments for the α-crystallin subunits. By measuring reactivity of sulphydryl groups to various reagents the observation was made that three classes of αA subunits exist, with respect to their position in the aggregate, namely surface exposed, partially exposed and totally buried [106]. This finding was later supported by a surface probing study in which α-crystallin lysine residues were modified by citraconic anhydride [107].

Bifunctional crosslinking experiments revealed a gradual decrease in intensity going from monomers to higher oligomers, suggesting that α-crystallin aggregates are not likely to be built up of smaller clusters such as trimers or tetramers. Not all subunits could be linked to each other, probably because some subunits are buried in the anterior of the aggregates [105, 108].

Electron microscopy, sedimentation analysis and small-angle X-ray scattering demonstrated that both native and reassociated α-crystallin are heterogeneous populations of spherical or slightly ellipsoidal molecules with diameters of ~15 nm for native α-crystallin and ~10 nm for reassociated α-crystallin [92, 109]. Partial dissociation in urea pointed to a stepwise dissociation of subunits, implicating the existence of multiple layers [105]. Limited *in vitro* proteolysis experiments revealed that all or nearly all αB-crystallin subunits are cleaved, suggesting that most B-chains are surface exposed and are likely to be in equivalent positions [110]. After αB-crystallin cleavage, αA-crystallin subunits are cleaved, but not at the same rate, indica-


A
1
2
3
4
B
1
2
3
C
1
2
3
D
1
2

ting that the αA subunits are in different environments. The above mentioned observations finally led to the three-layer model for the quaternary structure of α-crystallin (Fig. 1A). In this model the α-crystallin aggregate consists of three concentric layers of subunits. The core consists of 13 A chains, arranged in the way shown in Fig. 1A-1. The next 14 chains, 10 A and 4 B chains, may form a layer in between (Fig. 1A-2), while the remaining chains, 8 A and 8 B chains, are located in the outer layer (Fig. 1A-3). This model holds 43 subunits, with places still unoccupied in the outer layer; with all open places occupied (Fig. 1A-4), the model contains 51 subunits. This model explains the observed different environments for the subunits. It also accounts for the molecular weight of $1\text{-}1.1 \times 10^6$ found for nuclear α-crystallin, while the larger aggregates are supposed to be the result of linear polymerization [105].

By means of sedimentation analysis and electron microscopy the effects of alkaline pH, ionic strength, temperature and calcium ion concentration on the quaternary structure were studied [111]. A complex hypothetical scheme of transitions of bovine α-crystallin, caused by changes in these physicochemical parameters, was introduced. In this model the transitions can be easily explained with the three-layer model, being the result of partial dissociation of the consecutive layers. To endorse the three-layer model several other experiments, like fluorescence and near UV CD measurements, were done [112]. The spectroscopic signals parallelled changes in sedimentation coefficient upon increase of denaturant concentration, which was explained in terms of aromatic residues getting exposed upon dissociation to 12S intermediates. This suggests the existence of a two-layer 12S intermediate and thus supports the three-layer model.

Although the evidence for the three-layer model seems to be rather convincing, it was questioned by Thomson and Augusteyn [113, 114]. The three-layer model is based on the assumption that the at low temperature isolated α_c-crystallin, the 600-

Fig. 1: Models for the quaternaty structure of α-crystallin. *Panel A:* Three-layer model of Bindels *et al.* [105]. The core (A-1) is composed of 13 A chains (white spheres). The second layer (A-2) contains 14 not fully solvent-exposed subunits; 10 A chains and 4 B chains (black spheres). The outer layer (A-3) has 8 A and 8 B chains that are maximally solvent exposed. This model holds 43 subunits, corresponding with the molecular mass of α_c-crystallin (± 800 kDa) of α-crystallin. The outer layer contains open places that can be filled with additional subunits (A-4), resulting in a total of 51 subunits. *Panel B:* Three-layer model of Tardieu *et al.* [116], seen from a three-fold axis. The first layer (B-1) is made up of 12 A chains (grey spheres). The second layer (B-2) accomodates 24 subunits (white spheres), and the 12 equivalent sites of the third layer (B-3) are filled (black spheres) to yield a 48 subunit particle. This model is symmetrical and has a limited number of different subunit-subunit contacts. Two types of sites are present in the second and third layers, so two types of subunits may be accomodated in these layers. *Panel C:* Micellar three-layer model of Walsh *et al.* [104]. The innermost layer (C-1) is a micelle, consisting of 12 subunits, arranged in cuboctahedral symmetry. The apolar region (black) is directed inward, constituting a hydrophobic core. A second layer (C-2) consists of six subunits, directing their apolar faces toward the hydrophobic core. These two layers constitute a micelle-like structure with octahedral symmetry. The third layer (C-3) adds more subunits up to a total of not more than 24. The model, exhibiting cuboctahedral symmetry contains 42 subunits, corresponding to the molecular mass of α_c-crystallin (±800 kDa). *Panel D:* Quaternary structure model by Wistow [139]. Two possible isoforms of α-crystallin are presented: the cube (D-1) and the rhombic dodecahedron (D-2). In both isoforms each face consists of the C-terminal domains of four subunits (forming a tetramer), whereas the N-terminal domains are directed towards the interior. Each domain (indicated by a peanut-shape) in the tetramer interacts through identical a-b interactions. Tetramers associate by identical c-d interactions. The cube has 6 faces and hence 24 subunits. The rhombic dodecahedron has 12 faces and consists of 48 subunits.

1000 kDa aggregate, is the *in vivo* form of α-crystallin. Dissociation of α_c at low urea concentrations resulted in a relatively stable smaller aggregate, considered to represent the inner two layers [112]. Thomson and Augusteyn, however, considered the low molecular mass aggregate, α_m as the native *in vivo* situation. Their point of view was based on studies in which it was shown that at 37°C, better approaching the *in vivo* situation, α-crystallin is isolated as a 12S (320,000 Da) particle, whereas at 5°C it is isolated as a 17S (635,000 Da) particle. Additionally, they showed that dissociation of α_m-crystallin is completely reversible, which was interpreted as an indication that α_m is the native state. However, van den Oetelaar *et al.* showed that at 37°C, but under more physiological conditions concerning pH and ionic strength, still the larger aggregates (~800 kDa) are isolated [115].

The controversy, which one of the oligomers, α_c or α_m, is the *in vivo* form of α-crystallin, prompted Tardieu and co-workers to reexamine the three-layer model [116]. By means of X-ray and quasi electric light scattering they measured several hydrodynamic parameters of α-crystallin particles under varying conditions (pH, ionic strength and temperature]. The three-layer model, based on α_c being the native form of α-crystallin, appeared compatible with the experimental data. By applying basic principles of symmetry and by minimizing the number of different contacts between protein subunits, a three-layer model with tetrahedral symmetry was built (Fig. 1B). The core consists of 12 (Fig 1B-1), the second and third layer of 24 subunits each (Fig 1B-2, 3). The second and third layers possess two types of sites and can thus accomodate two types of subunits, αA and αB. The restrictions to minimize the amount of different types of subunit contacts and to maximize the amount of local contacts stabilizing the final structure, leads inevitably to probabilities of occupancy for the sites in outer layers. This accounts for the intrinsic polydispersity of α-crystallin and the fact that physicochemical parameters influence the aggregate size. The loss of subunits from α_c by changing physicochemical conditions is considered as an *in vitro* artefact, due to non-physiological conditions.

On basis of various experiments Augusteyn and co-workers disagreed with the three-layer model [114, 117-120]. They argued that the conclusion of Bindels and Siezen that the changes occurring between 1 and 4 M urea represent the progressive removal of a middle layer of subunits [105] was invalid [114]. Later, this was admitted by Bindels *et al.* [121]. Additionally, Augusteyn and co-workers demonstrated that there is only one class of sulphydryl groups in foetal α-crystallin [117], but that conformational changes in the protein during ageing give rise to the heterogeneity within this class, as was observed by Siezen *et al.* [106]. Further, they showed that α-crystallin particles can be constructed using any combination of purified A and B chains from any species [118]. In view of these observations and the similarities in sequences [122], immunochemical properties [119], and the microenvironments of aromatic and other amino acids [117, 120], they assumed that αA and αB have similar three-dimensional structures and consequently can occupy the same sites in the aggregate. Indeed, Hendriks *et al.* provided evidence that all three subunits in rat α-crystallin occupy equivalent positions [123]. Based on the above, a dodecamer displaying tetrahedral symmetry was suggested for the arrangement of subunits. This is consistent with the equivalence of subunits, but fails to explain the variations in size of the particle [118].

To account for the facts that the α-crystallin aggregate is polydisperse and its size distribution is dependent on physicochemi-

cal parameters [111, 113, 124], Augusteyn and Koretz proposed a flexible micelle structure with equivalent positions for all subunits [125]. By tryptophan fluorescence quenching studies it was shown that most likely, the N-terminal region is situated close to the center of the aggregate [120, 126]. Hydrophilicity profiles [127] suggested that the N-terminal domain is hydrophobic, whereas the C-terminal domain is more hydrophilic. This suggests that the N-terminal domain represents the hydrophobic end of the amphiphilic subunits, necessary for the formation of micelles. It should be noted, however, that the N-terminal domain is predicted to be less hydrophobic when another prediction method is used (Fig. 2). The possible existence of α-crystallin micelles was recently validated by means of hydrostatic pressure and surface tension experiments [128]. With increasing hydrostatic pressure the turbidity of an α-crystallin solution increases exponentially to a plateau, and the surface tension of α-crystallin in aqueous solution levels off with increasing protein subunit concentration, as with other amphipathic molecules aggregated into a micellar structure.

The existence of a flexible dynamic aggregate is also endorsed by the finding that the subunits are exchanged between aggregates under native conditions [129]. A variety of age-dependent changes occur in the eye lens. The electrolyte composition, which has been shown to influence the quaternary structure of α-crystallin [111, 115, 116], changes with age [130, 131]. α-Crystallin undergoes post-translational modifications during ageing [e.g. 2, 94-96, 132-134]. Due to dehydration of the bovine lens nucleus, the cellular protein concentration increases with age [135-137]. These changes might disturb the thermodynamic equilibrium of the existing α-crystallin population. By intermolecular exchange of subunits, new thermodynamically stable populations can be formed.

A new model for α-crystallin quaternary structure was recently introduced by Walsh *et al.* [104]. The proposed model of native α-crystallin has a three-layer structure in which the inner layer is a micelle containing 12 subunits arranged in cuboctahedral symmetry (Fig. 1C-1). The apolar region is directed inward constituting a hydrophobic core similar to a micelle and adding

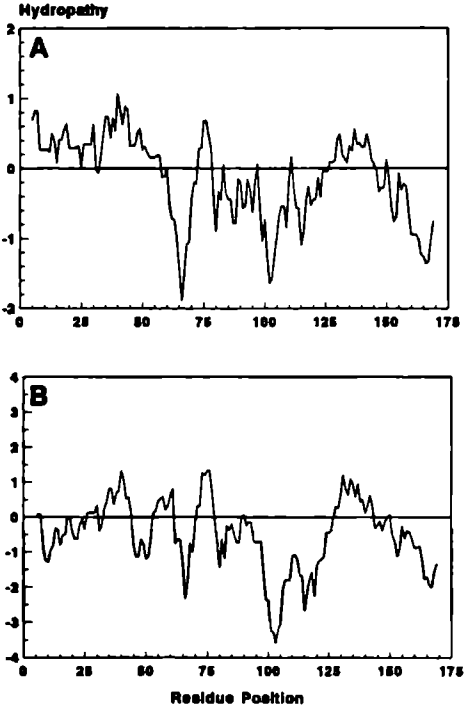


Fig. 2: Hydropathy patterns of bovine αA-crystallin, generated by two different methods. Average hydrophilicity values were calculated for each nonapeptide and plotted against the average sequence position of the nonapeptide. Negative values reflect hydrophilic regions, whereas positive values indicate hydrophobic regions. In panel A the prediction method of Hopp and Woods [149] was used, in panel B that of Kyte and Doolittle [150]. The two patterns differ in the region 1-50. The method of Hopp and Woods predicts this region to be hydrophobic, in contrast with the method of Kyte and Doolittle, which predicts this region to be predominantly hydrophilic

structural stability. A second layer consists of six subunits with their apolar faces directed towards the hydrophobic core (Fig.1C-2). The third layer adds more subunits up to total of 24 (Fig.1C-3). Subunit contacts in the third layer will be mostly polar-polar. Two thermal transitions were measured with differential scanning calorimetry. They might correspond with the dissociation of the third layer that reorients and forms a new two-layer structure, and the transition of the resulting two-layer aggregate into a more compact thermodynamically stable structure. This three-layer model has several advantages compared to the model proposed by Tardieu *et al.* [116]. It better accounts for the irreversibility of the $\alpha_c \rightarrow \alpha_m$ transition. Additionally, the molecular mass of the two inner layers of the new model better corresponds to that of α_m. The disadvantage of both models is that they are partly based on the reports that bovine α-crystallin contains three different classes of sulphydryl groups [106, 138]. This finding is contradicted by Augusteyn *et al.* [117].

The most recent model for α-crystallin quaternary structure was introduced by Wistow [139]. It is based on studies of Merck *et al.* [140] and on the assumption that among a variety of possible dynamic structures at least one stable structure is a roughly spherical aggregate of 800-900 kDa. Merck *et al.* demonstrated that the putative C-terminal domain and tail of αA-crystallin, which is highly conserved and maintains the closest similarity to the small HSPs, assembles into tetramers, whereas the more hydrophobic N-terminal domain forms less defined multimers. The ability of the C-terminal domain to tetramerize suggests that a tetramer is the fundamental building block for larger aggregates with four-fold symmetrical faces. One of these, the rhombic dodecahedron is proposed to be one of the stable forms of α-crystallin. In this model all subunits are in equivalent positions, although still three levels of interaction between subunits exist. The outside surface of rhombododecahedric α-crystallin consists of C-terminal domains, explaining its susceptibility towards several modifications [2, 94-96, 132-134]. Like in the micelle model, the core consists of mainly N-terminal domains, that probably interact rather unspecifically permitting variation in assembly. Finally, changing of physicochemical environments may lead to alternative quaternary structures, based on the same building block, e.g. the fourfold faced hexameric cube, with 24 subunits. This would explain the existence of smaller aggregates.

It is clear from the above that there is as yet absolutely no agreement on the quaternary structure of α-crystallin. This may be due to the proposed dynamic structure of α-crystallin [129, 139]. Moreover, the interpretation of structural data is seriously hampered by the influence of the isolation conditions on several physicochemical characteristics [111, 115], although a recent study demonstrates that the compositions of three different isolation buffers do not affect the properties of α-crystallin [141].

The *in vivo* situation is even more complex. The aggregate size of α-crystallin increases upon ageing [32, 142], a phenomenon that has been observed *in vitro* as well [143]. Furthermore, the α-crystallin aggregates are very heterogeneous, due to posttranslational modifications. These *in vivo* modifications include oxidation, deamidation, phosphorylation, racemization, chain cleavage, glycation, carbamylation and disulfide- and non-disulfide crosslinking [for reviews 93, 142, 144-146]. Many of these modifications cause changes in charge, size and solubility of α-crystallin, resulting in conformational changes, eventually leading to cataract formation [for reviews 142, 147, 148].

OUTLINE OF THIS THESIS

In order to better understand the functional and structural relationships between α-crystallin and the small HSPs , we compared several known features of α-crystallin with those of the mouse small HSP (chapter 2). To find out what is the importance of the evolutionarily conserved C-terminal domain of the members of the α-crystallin/small HSP family and whether this domain is responsible for the aggregate formation, several studies were undertaken. In chapter 3, the aggregation behaviour of the N-terminal domain and C-terminal domain and tail of αA-crystallin is described, while in chapter 4 the C-terminal domain and tail of three different members of the protein family are compared with regard to aggregation behaviour, secondary structure and heat protection capacity. The structure of αA-crystallin was further studied by site-directed mutagenesis (chapter 5). In chapter 6, it is reported that leakage of crystallins from the cataractous lens provokes an autoimmune response against most β- and γ-crystallins, but not against αA- and αB-crystallins, reflecting immunological tolerance because of the extralenticular occurrence of the latter.

Knowledge about the structure of α-crystallin will give insight into the interaction between proteins in the lens. It would moreover teach us about the interprotein interactions that play a role in the molecular chaperoning of proteins by α-crystallin and the small HSPs.

REFERENCES

1. Mörner, C. T. (1894) Hoppe Seyler's Z. Physiol. Chem. 18, 61-106.
2. de Jong, W. W. (1981), in Molecular and cellular biology of the eye lens (Bloemendal, H., ed.) pp. 221-278, Wiley, New York.
3. Stapel, S. O., Leunissen, J. A. M., Versteeg, M., Wattel, J. and De Jong, W. W. (1984) Nature 311, 257-259.
4. De Jong, W. W., Leunissen, J. A. M., Leenen, P. J. M., Zweers, A. and Versteeg, M. (1988) J. Biol. Chem. 263, 5141-5149
5. Ingolia, T. D. and Craig, E. A. (1982) Proc. Natl. Acad. Sci. USA 79, 2360-2364
6. Bhat, S. P. and Nagineni, C. N. (1989) Biochem. Biophys. Res. Comm. 158, 319-325.
7. Dubin, R. A., Wawrousek, E. F. and Piatigorsky, J. (1989) Mol. Cell. Biol. 9, 1083-1091.
8. Duguid, J. R., Rohwer, R. G. and Seed, B. (1988) Proc. Natl. Acad. Sci. USA 85, 5738-5742.
9. Kato, K., Shinohara, H., Kurobe, N., Goto, S., Inaguma, Y. and Ohshima, K. (1991) Biochim. Biophys. Acta 1080, 173-180.
10. Klemenz, R., Fröhli, E., Steiger, R. H., Schäfer, R. and Aoyama, A. (1991) Proc. Natl. Acad. Sci. USA 88, 3652-3656.
11. Piatigorsky, J. (1989) FASEB J. 3, 1933-1940.
12. Wistow, G. J. and Piatigorsky, J. (1988) Annu. Rev. Biochem. 52, 479-504.
13. Piatigorsky, J. and Wistow, G. J. (1989) Cell 57, 197-199.
14. De Jong, W. W., Hendriks, W., Mulders, J. W. M. and Bloemendal, H. (1989) Trends Biochem. Sci. 14, 365-368.
15. Doolittle, R. F. (1988) Nature 336, 18-19.
16. Lindquist, S. and Craig, E. A. (1988) Ann. Rev. Genet. 22, 631-677.
17. De Jong, W. W., Leunissen, J. A. M. and Voorter, C. E. M. (1993) Mol. Biol. Evol. 10, 103-116.
18. Kato, K., Shinohara, H., Kurobe, N., Inaguma, Y., Shimizu, K. and Ohshima, K. (1991) Biochim. Biophys. Acta 1074, 201-208.
19. Ellis, R. J. and Van der Vies, S. M. (1991) Annu. Rev. Biochem. 60, 321-347.
20. Gething, M. -J. and Sambrook, J. (1992) Nature 355, 33-45.
21. Berger, E. M. and Woodward, M. P. (1983) Exp. Cell Res. 147, 437-442.
22. Hickey, E., Brandon, S. E., Potter, R., Stein, G., Stein, J. and Weber, L. A. (1986)

Nucleic Acids Res. 14, 4127-4145.
23. Landry, J., Chretien, P., Lambert, H., Hickey, E. and Weber, L. A. (1989) J. Cell Biol. 109, 7-15.
24. Bentley, N. J., Fitch, I. T. and Tuite, M. F. (1992) Yeast 8, 95-106.
25. Petko, L. and Lindquist, S. (1986) Cell 45, 885-894.
26. Susek, R. E. and Lindquist, S. L. (1989) Mol. Cell. Biol. 9, 5265-5271.
27. Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M. and Geiger, B. 1991) J. Cell Biol. 114, 255-261
28. Mansfield, M. A. and Key, J. L. (1987) Plant Physiol. 84, 1007-1017.
29. van den Heuvel, R., Hendriks, W., Quax, W. and Bloemendal, H. (1985) J. Mol. Biol. 185, 273-284.
30. Wistow, G. (1985) FEBS Lett. 181, 1-6.
31. Merck, K. B., Groenen, P. J. T. A., Voorter, C. E. M., De Haard-Hoekman, W. A., Horwitz, J., Bloemendal, H. and De Jong, W. W. (1993) J. Biol. Chem. 268, 1046-1052.
32. Spector, A., Li, L. -K., Augusteyn, R. C., Schneider, A. and Freund, T. (1971) Biochem. J. 124, 337-343.
33. Clauwaert, J., Ellerton, H. D., Koretz, J. F., Thomson, K. and Augusteyn, R. C. 1989) Curr. Eye Res. 8, 397-403.
34. Collier, N. C., Heuser, J., Levy, M. A. and Schlesinger, M. J. (1988) J. Cell Biol. 106, 1131-1139.
35. Arrigo, A. -P., Suhan, J. P. and Welch, W. J. (1988) Mol. Cell. Biol. 8, 5059-5071.
36. Chiesi, M., Longoni, S. and Limbruno, U. (1990) Mol. Cell. Biol. 97, 129-136.
37. Nover, L., Scharf, K. -L. and Neumann, D. (1989) Mol. Cell. Biol. 9, 1298-1308.
38. Collier, N. C. and Schlesinger, M. J. (1986) J. Cell Biol. 103, 1495-1507.
39. Inaguma, Y., Shinohara, H., Goto, S. and Kato, K. (1992) Biochem. Biophys. Res. Comm. 182, 844-850.
40. Voorter, C. E. M., Wintjes, L., Bloemendal, H. and De Jong, W. W. (1992) FEBS Lett. 309, 111-114.
41. Zantema, A., Verlaan-de Vries, M., Maasdam, D., Bol, S. and van der Eb, A. (1992) J. Biol. Chem. 267, 12936-12941.
42. Kato, K., Shinohara, H., Goto, S., Inaguma, Y., Morishita, R. and Asano, T. (1992) J. Biol. Chem. 267, 7718-7725.
43. Arrigo, A. -P. and Paulì, D. (1988) Exp. Cell Res. 175, 169-183.
44. Longoni, S., Lattonen, S., Bullock, G. and Chiesi, M. (1990) Mol. Cell. Biol. 97, 121-128.
45. Behlke, J., Lutsch, G., Gaestel, M. and Bielka, H. (1991) FEBS Lett. 288, 119-122.
46. Quax-Jeuken, Y., Quax, W., van Rens, G. L. M., Meera Khan, P. and Bloemendal, H. (1985) Cytogenet. Cell Genet. 40, 727-728.
47. Ngo, J. T., Klisak, I., Dubin, R. A., Piatigorsky, J., Mohandas, T., Sparkes, R. S. and Bateman, B. (1989) Genomics 5, 665-669.
48. Morimoto, R. I., Tissières, A. and Georgopoulos, C. (1990) Stress proteins in Biology and Medicine, Cold Spring Harbor Laboratory Press, New York.
49. DasGupta, S., Hohman, T. C. and Carper, D. (1992) Exp. Eye Res. 54, 461-470.
50. Rossi, J. M. and Lindquist, S. (1989) J. Cell Biol. 108, 425-439.
51. Bienz, M. (1984) Proc. Natl. Acad. Sci. USA 81, 3138-3142.
52. Hayward, J. R., Coffer, A. I. and King, R. J. B. (1990) J. Steroid Biochem. Mol. Biol. 37, 513-519.
53. Klemenz, R., Fröhli, E., Aoyama, A., Hoffmann, S., Simpson, R. J., Moritz, R. L. and Schäfer, R. (1991) Mol. Cell. Biol. 11, 803-812.
54. Aoyama, A., Fröhli, E., Schäfer, R. and Klemenz, R. (1993) Mol. Cell. Biol. (In Press)
55. Voorter, C. E. M., Mulders, J. W. M., Bloemendal, H. and De Jong, W. W. (1986) Eur. J. Biochem. 160, 203-210.
56. Chiesa, R., Gawinowicz-Kolks, M. A., Kleiman, N. J. and Spector, A. (1987) Biochem. Biophys. Res. Comm. 144, 1340-1347.
57. Chiesa, R. and Spector, A. (1989) Biochem. Biophys. Res. Comm. 162, 1494-1501.
58. Mann, E , McDermott, M. J., Goldman, J., Chiesa, R. and Spector, A. (1991) FEBS Lett. 294, 133-136.
59. Arrigo, A. -P. and Welch, W. J. (1987) J. Biol. Chem. 262, 15359-15369.
60. Saklatvala, J., Kaur, P. and Guesdon, F. (1991) Biochem. J. 277, 635-642.
61. Landry, J., Chretien, P., Laszlo, A. and Lambert, H. (1991) J. Cell. Physiol. 147, 93-101.
62. Regazzi, R., Eppenberger, U. and Fabbro, D. (1988) Biochem. Biophys. Res. Comm. 152, 62-68.
63. Mendelsohn, M. E., Zhu, Y. and O'Neill,

S. (1991) Proc. Natl. Acad. Sci. USA 88, 11212-11216.
64. Guesdon, F. and Saklatvala, J. (1991) J. Immunol. 147, 3402-3407.
65. Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A. and Anderson, C. W. (1992) J. Biol. Chem. 267, 794-803.
66. Scotting, P., McDermott, H. and Mayer, R. J. (1991) FEBS Lett. 285, 75-79.
67. Anson, J. F., Laborde, J. B., Pipkin, J. L., Hinson, W. G., Hanser, D. K., Sheehan, D. M and Young, J. F. (1991) Teratology 44, 19-28.
68. Mayer, R. J., Arnold, J., Laszlo, L., Landon, M. and Lowe, J. (1991) Biochim. Biophys. Acta 1089, 141-157.
69. Iwaki, T., Wisniewski, T., Iwaki, A., Corbin, E., Tomokane, N., Tateishi, J. and Goldman, J. E. (1992) Am. J. Pathol. 140, 345-356.
70. Iwaki, T. and Tateishi, J. (1991) Am. J. Pathol. 139, 1301-1308.
71. Iwaki, T., Iwaki, A., Miyazono, M. and Goldman, J. E. (1991) Cancer 68, 2230-2240.
72. Lowe, J., McDermott, H., Pike, I., Spendlove, I., Landon, M. and Mayer, R. J. 1992) J. Pathol. 166, 61-68.
73. Tomokane, N., Iwaki, T., Tateishi, J., Iwaki, A. and Goldman, J. E. (1991) Am. J. Pathol. 138, 875-885.
74. Thor, A., Benz, C., Moore II, D., Goldman, E., Edgerton, S., Landry, J., Schwartz, L., Mayall, B., Hickey, E. and Weber, L. A. (1991) J. Natl. Cancer Inst. 83, 170-178.
75. Zantema, A., de Jong, E., Lardenoije, R. and van der Eb, A. J. (1989) J. Virol. 63, 3368-3375.
76. Kato, M., Herz, E., Kato, S. and Hirano, A. (1992) Acta Neuropathol. 83, 420-422.
77. Mulders, J. W. M., Stokkermans, J., Leunissen, J. A. M., Benedetti, E. L., Bloemendal, H. and De Jong, W. W. (1985) Eur. J. Biochem. 152, 721-728.
78. Ifeanyi, F. and Takemoto, L. J. (1990) Curr. Eye Res. 9, 259-265.
79. Bloemendal, H., Berbers, G. A. M., De Jong, W. W., Ramaekers, F. C. S., Vermorken, A. J. M , Dunia, I. and Benedetti, E L. (1984), in Human cataract formation, Ciba Fnd. Symposium 106 (pp. 177-186, Pitman, London
80. Fitzgerald, P. G. and Graham, D. (1991) Curr. Eye Res. 10, 417-436.
81. Grimm, B., Ish-Shalom, D., Even, D., Glaczinski, H., Ottersbach, P., Kloppstech, K. and Ohad, I. (1989) Eur. J. Biochem. 182, 539-546.
82. Adamska, I. and Kloppstech, K. (1991) Eur. J. Biochem. 198, 375-381.
83. Lim, L., Hall, C., Leung, T. and Whatley, S. (1984) Biochem. J. 224, 677-680.
84 Horwitz, J. (1992) Proc. Natl. Acad. Sci. USA, 89, 10449-10453.
85. Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) J. Biol. Chem. (In Press)
86. Delaye, M. and Tardieu, A. (1983) Nature 302, 415-417.
87. Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, I., Turnell, B. and Wistow, G. (1981) Nature 289, 771-777.
88. Chirgadze, Y. N., Nevskaya, N. A., Fomenkova, N. P., Nikonov, S. V., Sergeev, Y. V., Brazhnikov, E. V., Garber, M. B., Lunin, V. Y., Urzumtsev, A. P. and Vernoslova, E. A. (1986) Dokl. Akad. Nauk S. S. S. R. 290, 492-495.
89. White, H. E., Driessen, H. P. C., Slingsby, C., Moss, D. S. and Lindley, P. F. 1989) J. Mol. Biol. 207, 217-235.
90. Bax, B., Lapatto, R., Nalini, V., Driessen, H., Lindly, P. F., Mahadevan, D., Blundell, T. L. and Slingsby, C. (1990) Nature 347, 776-780.
91. van Rens, G. L. M., Driessen, H. P. C., Nalini, V., Slingsby, C., De Jong, W. W. and Bloemendal, H. (1991) Gene 102, 179-188.
92. Siezen, R. J., Bindels, J. G. and Hoenders, H. J. (1978) Eur. J. Biochem. 91, 387-396.
93. De Jong, W. W., Mulders, J. W. M., Voorter, C. E. M., Berbers, G. A. M., Hoekman, W. A. and Bloemendal, H. (1988), in Advances in posttranslational modifications of proteins and ageing (Zappia, V., Galletti, P., Porta, R and Wold, F. , eds.) pp. 95-108, Plenum Press, New York
94. Groenen, P. J. T. A., Bloemendal, H. and De Jong, W. W. (1992) Eur. J. Biochem. 205, 671-674.
95. Roquemore, E. P., Dell, A., Morris, H. R., Panico, M., Reason, A. J., Savoy, L. -A., Wistow, G. J., Zigler, J. S.,Jr., Earles, B. J. and Hart, G. W. (1992) J. Biol. Chem. 267, 555-563.
96. Voorter, C. E. M., De Haard-Hoekman, W. A., Roersma, E. S., Meyer, H. E., Bloemendal, H. and De Jong, W. W. (1989)

FEBS Lett. 259, 50-52.
97. Siezen, R. J. and Argos, P. (1983) Biochim. Biophys. Acta 748, 56-67.
98. Liang, J. N. and Chakrabarti, B. (1982) Biochemistry 21, 1847-1852.
99. Li, L. -K. and Spector, A. (1974) Exp. Eye Res. 19, 49-57.
100. Siezen, R. J. (1981) FEBS Lett. 133, 1-8.
101. Argos, P. and Siezen, R. J. (1983) Eur. J. Biochem. 131, 143-148.
102. Siezen, R. J., Owen, E. A., Kubota, Y. and Ooi, T. (1983) Biochim. Biophys. Acta 748, 48-55.
103. Maiti, M., Kono, M. and Chakrabarti, B. (1988) FEBS Lett. 236, 109-114.
104. Walsh, M. T., Sen, A. C. and Chakrabarti, B. (1991) J. Biol. Chem. 266, 20079-20084.
105. Bindels, J. G., Siezen, R. J. and Hoenders, H. J. (1979) Ophthalmic Res. 11, 441-452.
106. Siezen, R. J., Coenders, F. G. and Hoenders, H. J. (1978) Biochim. Biophys. Acta 537, 456-465.
107. Bindels, J. G., Misdom, L W. and Hoenders, H. J. (1985) Biochim. Biophys. Acta 828, 255-260.
108. Siezen, R. J., Bindels, J. G. and Hoenders, H. J. (1980) Eur. J. Biochem. 107, 243-249.
109. Siezen, R. J. and Berger, H. (1978) Eur. J. Biochem. 91, 397-405.
110. Siezen, R. J. and Hoenders, H. J. (1979) Eur. J. Biochem. 96, 431-440.
111. Siezen, R. J., Bindels, J. G. and Hoenders, H. J. (1980) Eur. J. Biochem. 111, 435-444.
112. Siezen, R. J. and Bindels, J. G. (1982) Exp. Eye Res. 34, 969-983.
113. Thomson, J. A. and Augusteyn, R. C. (1983) Exp. Eye Res. 37, 367-377.
114. Thomson, J. A. and Augusteyn, R. C. (1984) J. Biol. Chem. 259, 4339-4345.
115. van den Oetelaar, P. J., Clauwaert, J., van Laethem, M. and Hoenders, H. J. 1985) J. Biol. Chem. 260, 14030-14034.
116. Tardieu, A., Laporte, D , Licinio, P., Krop, B. and Delaye, M. (1986) J. Mol. Biol. 192, 711-724.
117. Augusteyn, R. C., Hum, T. P., Putilin, T. P. and Thomson, J. A. (1987) Biochim. Biophys. Acta 915, 132-139.
118. Thomson, J. A. (1985) A model for multisubunit protein assemblies, PhD Thesis, University of Melbourne,.
119. Butler, D. M. and Augusteyn, R. C. (1986) Curr. Eye Res. 5, 225-229.
120. Augusteyn, R. C., Putilin, T. P. and Seifert, R. (1988) Curr. Eye Res. 7, 237-245.
121. Bindels, J. G., van den Oetelaar, P. J. and Hoenders, H. J. (1986) J. Liq. Chromatogr. 9, 1297-1315.
122. De Jong, W. W. (1981), in Molecular and Cellular Biology of the Eye Lens (Bloemendal, H., ed.) pp. 279-326, Wiley, New York
123. Hendriks, W., Weetink, H., Voorter, C. E. M., Sanders, J., Bloemendal, H. and De Jong, W. W. (1990) Biochim. Biophys. Acta 1037, 58-65.
124. Augusteyn, R. C., Lankovsky, T. and Stevens, A. (1983) Exp. Eye Res. 37, 367-377.
125. Augusteyn, R. C. and Koretz, J. F. (1987) FEBS Lett. 222, 1-5.
126. Phillips, S. R., Wilson, L. J. and Borkman, R. F. (1986) Curr. Eye Res. 5, 611-619.
127. Puri, N., Augusteyn, R. C., Owen, E. A. and Siezen, R. J. (1983) Eur. J. Biochem. 134, 321-326.
128. Radlick, L. W. and Koretz, J. F. (1992) Biochim. Biophys. Acta 1120, 193-200.
129. van den Oetelaar, P. J., van Someren, P. F. H. M , Thomson, J. A., Siezen, R. J. and Hoenders, H. J. (1990) Biochemistry 29, 3488-3493.
130. Rink, H. and Twenhöven, H. (1985) Ophthalmic Res. 17, 321-324.
131. Bloemendal, H., Hockwin, O., Hoenders, H. J., Ohrloff, C. and Rink, H. (1985), in Biochemie des Auges (Hockwin, O., ed.) pp. 82-109, Enke Verlag, Stuttgart
132. Chiesa, R., Gawinowicz-Kolks, M. A., Kleiman, N. J. and Spector, A. (1987) Curr. Eye Res. 6, 539-542.
133. Bloemendal, H. (1981), in Molecular and cellular biology of the eye lens (Bloemendal, H., ed.) pp. 1-47, Wiley, New York
134. Harding, J. J. and Crabbe, M. J. C. (1984), in The Eye Vol. 1B (Davson, H., ed.) pp. 207-492, Academic Press, New York
135. Rink, H. (1977) Interdiscip. Top. Gerontol. 12, 271-277.
136. Rink, H., Muenninghoff, J. and Hockwin, O. (1977) Ophthalmic Res. 9, 129-135.
137. Hockwin, O., Rast, F., Rink, H., Muenninghoff, J. and Twenhöven, H. (1978) Interdiscip. Top. Gerontol. 13, 102-108.
138. Spector, A. and Zorn, M. (1967) J. Biol. Chem. 242, 3594-3600.
139. Wistow, G. J. (1993) Exp. Eye Res. (In Press)
140. Merck, K. B., De Haard-Hoekman, W. A., Oude-Essink, B. B., Bloemendal, H. and De Jong, W. W. (1992) Biochim. Biophys. Acta

1130, 267-276.
141. Augusteyn, R. C., Parkhill, E. M. and Stevens, A. (1992) Exp. Eye Res. 54, 219-228.
142. Hoenders, H. J. and Bloemendal, H. (1981), in Molecular and cellular biology of the eye lens (Bloemendal, H., ed.) pp. 279-326, Wiley, New York.
143. Bloemendal, M., van Amerongen, H., Bloemendal, H. and van Grondelle, R. 1989) Eur. J. Biochem. 184, 427-432.
144. Harding, J. J and Dilley, K. J. (1976) Exp. Eye Res. 22, 1-73.
145. Zigler, J. S.,Jr. and Goosey, J. (1981) Trends Biochem. Sci. 6, 133-136.
146. Hoenders, H. J. and Bloemendal, H. (1983) J. Gerontol. 38, 278-286.
147. Harding, J. J. and Crabbe, M. J. C. (1984), in The Eye (Davson, H., ed.) pp. 207-492, Academic Press, New York
148. Harding, J. (1991) Cataract-biochemistry, epidemiology and pharmacology, Chapman & Hall, London.
149. Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132
150. Hopp, T. D. and Woods, K. P. (1981) Proc. Natl. Acad. Sci. USA 78, 3824-3828.

CHAPTER 2

Structural and functional similarities of bovine α-crystallin and mouse small heat shock protein.

A family of chaperones

Structural and functional similarities of bovine α-crystallin and mouse small heat shock protein. A family of chaperones.

α-Crystallin, composed of the subunits αA and αB, is a major vertebrate eye lens protein, accomplishing a structural role in maintaining lens stability and transparency. Both subunits also occur in low amounts outside the lens, where their precise function is unknown. They are structurally related to the small heat shock proteins (HSPs[1]), and increasing evidence indicates that they have also functional similarities with the small HSPs. In order to extend our insight into these structural and functional relationships, the mouse small HSP (HSP25) was compared with bovine α-crystallin, with respect to several known properties of the latter. We show that α-crystallin and HSP25 resemble each other in secondary structure, and have the same stability towards urea dissociation at pH 7.0. Mixed polymers can be formed from any combination of αA-crystallin, αB-crystallin and HSP25 subunits. Furthermore, we demonstrate that HSP25, like α-crystallin, can function as a molecular chaperone, by suppressing heat-induced aggregation of other proteins, and is an efficient inhibitor of elastase. Finally, HSP25 is found to be a substrate for protein crosslinking by tissue-type transglutaminase, like αB-crystallin. Our results thus corroborate that α-crystallin and the small HSPs have comparable functions, probably being involved in the protection of other proteins under conditions of stress

INTRODUCTION

Ten years ago it was discovered that the *Drosophila* small heat shock proteins are homologous with the vertebrate eye lens protein α-crystallin [1]. This led to the suggestion that α-crystallin has evolved from a heat shock protein-like ancestor [1, 2]. While fully enigmatic at the time, several recent findings shed light on this evolutionary transition of a ubiquitous stress protein into a structural lens protein [3]. Most revealing has been the discovery that the two homologous subunits of α-crystallin, αA and αB, until then thought to be strictly lens-specific, both occur constitutively outside the eye. αB-Crystallin reaches considerable levels in heart, striated muscle and kidney, and is present in many other tissues [4-7]. Small amounts of αA-crystallin are found in spleen and thymus [8]. Equally important is the demonstration that αB-crystallin behaves in several respects like the mammalian small HSPs. The expression of αB-crystallin is induced upon stress [9-11] and under certain pathological conditions [12-17]. After stress it relocalizes from the cytoplasm towards the nucleus [9, 11, 16, 18]. Apart from the sequence homology, which is most pronounced in the C-terminal halves of α-crystallin and small HSPs, the proteins also resemble each other in their quaternary structure. Both form large complexes, usually between 400 and 800 kDa [19-22], which electron microscopically appear as 10 to 18 nm globular structures [23-27]. The aggregate size of the small heat shock proteins increases during stress [19, 28]. Similarly, amorphous αB-crystallin aggregates are formed after ischemia [16].

To enable a further comparison between α-crystallin and small HSPs, we analysed

[1] The abbreviations used are: HSP, heat shock protein; HSP25, mouse small HSP; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; Bistris, 2,2-Bis(hydroxymethyl)-2,2',2''-nitriloethanol.

some important structural and functional features of both proteins. These include comparison of secondary structure, dissociation in urea, ability to form mixed aggregates, ability to inhibit elastase [29] and the ability to be a substrate for transglutaminase [30]. In addition, we examined the ability of the mouse small HSP to suppress heat-induced aggregation of other proteins, a property which was recently shown for α-crystallin [31].

MATERIALS AND METHODS

Isolation of proteins

α-Crystallin was isolated from the water-soluble fraction of calf lens cortex by gel permeation chromatography [32]. αA- and αB-subunits were separated by ion-exchange chromatography on DEAE-cellulose [32]. Recombinant mouse HSP25 was expressed in *E.coli* BL21(DE3), using the expression clone pAK3038p25, as described by Gaestel *et al.* [33]. The mouse HSP25 was purified from the *E.coli* lysate essentially according to Gaestel *et al.* [34]. Recombinant bovine αA-crystallin was expressed in *E.coli* BL21(DE3), as described by Merck *et al.* [35]. αA-Crystallin was purified from the *E.coli* water-soluble fraction by ion-exchange chromatography on Fast Flow DEAE-cellulose (Pharmacia-LKB), applying a linear phosphate gradient (0.05 M to 0.40 M sodium phosphate, pH 6.8). Recombinant αA-crystallin was only used in the circular dichroism experiments. ßL-crystallin, used in the heat protection assay was obtained by gel filtration [32].

Circular dichroism measurements

These were performed using a Jasco Model 600 spectropolarimeter. A 0.25 mm or 0.50 mm pathlength cell was used.

Tryptophan fluorescence

Tryptophan fluorescence was measured on samples containing 50-100 μg of protein per ml in 50 mM Bistris (pH 6.0 or 7.0) and 0.1% β-mercaptoethanol. The concentration of urea in the preparations ranged from 0 to 7 M. After at least 2 h of incubation at room temperature, fluorescence emission spectra were recorded with a Hitachi F-3000 spectrofluorometer and associated data station, using a 1 cm pathlength quartz cuvette. The spectra were recorded over the 300-400 nm range, using an excitation wavelength of 295 nm, with a 5 nm excitation and a 3 nm emission slit. All spectra were corrected for the solvent used. Transition points were calculated as described by Pace *et al.* [36].

Reaggregation of pure and mixed polypeptides

Various proteins or protein mixtures, containing approximately equal amounts of each protein, were dissolved in phosphate-buffered 6 M urea (50 mM sodium phosphate, 50 mM NaCl, 1 mM EDTA, 0.02% β-mercaptoethanol, pH 7.2), with a final protein concentration of about 1 mg/ml. After 2 h of incubation at room temperature, the solutions were diluted to a final concentration of 1 M urea with the above mentioned phosphate buffer, containing protease inhibitors (2 μg/ml pepstatin A, 0.1 μmol/ml benzamidin, 1 μg/ml bacitracin, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 0.2 μM phenylmethylsulfonyl fluoride). The urea was removed by dialysis at 4°C against deionized water, containing 0.02% β-mercaptoethanol. After lyophilization, the renatured protein samples were subjected to gel permeation analysis or biotinylation and immunoprecipitation.

Gel permeation analysis
200-300 μg of the reaggregated proteins or protein mixtures were analysed by means of FPLC gel permeation chromatography, using a Superose 6 HR 10/30 prepacked column (Pharmacia-LKB). The column was equilibrated with 50 mM sodium phosphate (pH 7.5), 50 mM NaCl and 1 mM EDTA . The column was eluted at room temperature, at a flow rate of 0.5 ml/min and monitored by absorbance at 280 nm. High molecular weight markers from the Pharmacia gel filtration calibration kit were used.

Biotinylation and immunoprecipitation
Before immunoprecipitation, 100-300 μg of the reaggregated protein samples were labelled with biotin by incubation with 0.2 mM NHS-LC-biotin (Pierce) for 2 h at room temperature in 300 μl biotinylation buffer (0.1 M NH_4HCO_3, 0.1 M NaCl, pH 8.0). The reaction was stopped by addition of 3 μl 1 M Tris/HCl, pH 8.0. Immunoprecipitation was performed with polyclonal antibodies directed against mouse HSP25, bovine αA- and αB-crystallin coupled to protein A Sepharose beads (Pharmacia). To that end 100 μl of the polyclonal antisera were incubated with 200 μl of a 50% protein A Sepharose beads suspension in coupling buffer (10 mM Tris/HAc, pH 8.0, 0.5 M NaCl, 0.1% Nonidet P-40) during 2 h at 4°C in an end-over-end rotator. The beads were then washed 5 times with 1 ml immunoprecipitation buffer (50 mM Tris/HAc, pH 8.0, 0.5 M NaCl, 0.1% NP-40) and stored at 4°C. Before immunoprecipitation the protein samples, containing 10 μg of biotinylated protein, were clarified by centrifugation (20 min., 15,000 g, 4°C). The supernatants were incubated with 20 μl of the antibody-loaded protein A Sepharose beads, in the presence of protease inhibitors, in 1 ml of immunoprecipitation buffer, during 2 h at 4°C, in an end-over-end rotator. The protein A Sepharose beads were centrifuged and washed 3 times with immunoprecipitation buffer. The pellet was dissolved in 20 μl SDS-PAGE loading buffer and a 10 μl sample was resolved by SDS-PAGE [37] for subsequent streptavidin analysis.

Elastase inhibition assay
The activity of porcine pancreas elastase (Whatman) was monitored spectrophotometrically at 405 nm using the substrate N-methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-p-nitroanilide (Sigma). The assay mixture contained 2 μg elastase and 1.0 mM substrate in a 10 mM Tris buffer (pH 8.0). Inhibition was assessed after preincubation of elastase with inhibitor for 3 minutes, at room temperature, and initiating the reaction by the addition of substrate.

Heat protection assay
The capacity of HSP25 and the α-crystallin subunits to protect ßL-crystallin and yeast α-glucosidase (Sigma Chemical Co.) against heat-induced aggregation was performed as follows: The apparent absorption at 360 nm, due to scattering, was measured in a Beckman DU 70 spectrophotometer, equipped with a 6-cell-holder accessory, and a Peltier temperature controller accessory. The temperature of the samples in the cells was measured by inserting a small bead-thermocouple inside one of the 6 cells in the holder. Teflon-stoppered, black-walled, 10 mm-pathlength, 2 mm wide cells were used. In each experiment the total volume in the cell was 400 μl. The scattering of each cell was sampled and recorded automatically every 20 sec. Proteins and buffers were mixed in the cell at room temperature. The average time required for the mixture inside the cell to reach the predetermined temperature of the cell holder was approximately 5 min.

Determination of transglutaminase substrates

To study the lysine-substrate capacity in the transglutaminase-mediated crosslinking, purified α-crystallin and HSP25 were treated with guinea-pig liver transglutaminase (Sigma), in the presence of a biotinylated amine-acceptor hexapeptide, and analysed essentially as described by Groenen *et al.* [30].

Miscellaneous methods

Digestion of α-crystallin and recombinant HSP25 with carboxypeptidase B (Merck) was performed as described by Siezen *et al.* [38]. Alkaline urea gel electrophoresis was carried out at pH 8.5 [32]. Immunoblot analysis was performed according to Mulders *et al.* [39]. For Western blotting, the polyclonal antisera against αA-, αB-crystallin and recombinant HSP25 (the latter obtained from A. Zantema [40]) were used at a 1:2000 dilution. The peroxidase-conjugated swine anti-rabbit immunoglobulins (SwaRPO, Dakopatts) were used at a 1:200 dilution. Streptavidin blot analysis was carried out as described by Groenen *et al.* [30].

RESULTS

Secondary structure of α-crystallin and HSP25

Although the tertiary structure of α-crystallin is not yet known, secondary structure data are available. Circular dichroism experiments have revealed that α-crystallin has mainly ß-pleated sheet conformation and contains very little α-helix conformation [41, 42]. To compare the secondary structures of α-crystallin and the small HSP25, far UV CD measurements were performed on α-crystallin isolated from calf cortex, recombinant αA-crystallin and recombinant HSP25. These spectra are shown in Fig. 1. The profile of all three spectra is very similar, with a single minimum at ~217 nm. These spectra are typical of predominantly ß-sheet proteins [43]. The intensity differences among the three profiles are most probably due to differences in protein concentrations. These results suggest that the secondary structure of recombinant αA and the recombinant HSP25 are very similar to that of the native α-crystallin.

Stability of αA-crystallin, αB-crystallin and HSP25 aggregates

The quaternary structure of α-crystallin and its urea stability have been studied extensively [e.g. 42, 44]. The small HSPs and α-crystallin form similar aggregates, with respect to size [19-21, 28] and particle shape [23-27, 45]. To obtain an additional indication that α-crystallins and HSP25 have similar structural properties, their stability was studied by measuring

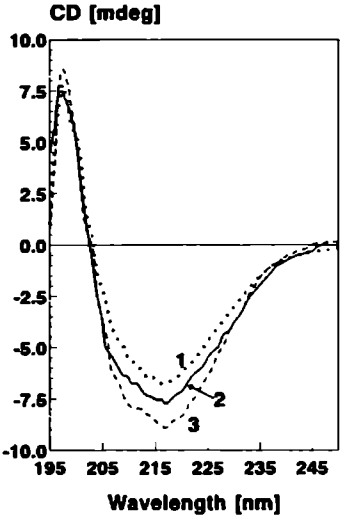


Fig. 1: Far UV circular dichroism spectra of α-crystallin and HSP25. Curve 1; calf lens α-crystallin, protein concentration ~0,7 mg/ml, pathlength 0.25 mm. Curve 2; recombinant HSP25, protein concentration ~0,7 mg/ml, pathlength 0.25 mm. Curve 3; recombinant αA-crystallin, protein concentration ~0,35 mg/ml, pathlength 0.5 mm. Each spectrum is the average of 8 scans.

the tryptophan fluorescence wavelength maxima as a function of urea concentration. Since inconsistent results were obtained earlier for the transition points of αB-crystallin [42, 44], most probably due to the use of buffers with different pH, the measurements in this study were carried out at three different pH values. Two distinct isolates for each polypeptide were used to measure urea transition curves.

Because the transition points at pH 7.0 of αA-crystallin, αB-crystallin and HSP25 are 2.54, 2.53 and 2.52 M urea, respectively (Fig. 2A) it may be concluded that all three proteins are equally stable. The shape of the αA-crystallin transition curve is somewhat flatter than those of αB-crystallin and HSP25, but still has the same transition point. At lower pH the stability of αB-crystallin decreases significantly. The transition point at pH 6.0 drops to 1.54 M (Fig. 2B). The stability of αA-crystallin is not decreased at lower pH, whereas the stability of HSP25 is slightly, but not significantly, reduced at lower pH. Apparently, the earlier observed discrepancy between the measured stabilities of αB-crystallin [42, 44] is due to the different pH values used in these two studies.

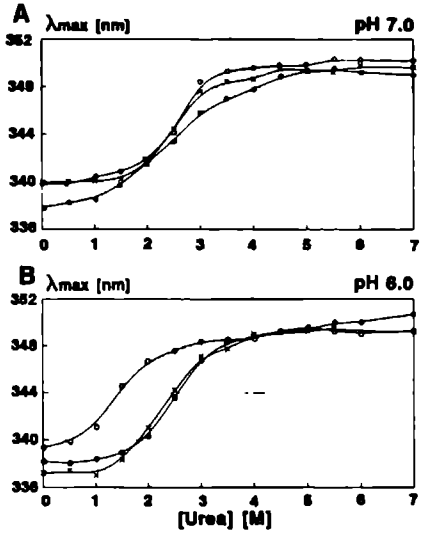


Fig. 2: Urea denaturation curves of αA-crystallin(●), αB-crystallin (○) and HSP25 (×). Tryptophan fluorescence emission maxima were measured as a function of urea concentration in 50 mM Bistris at pH 7.0 (A) and pH 6.0 (B). The transition points were determined as described in Materials and Methods.

Formation of mixed aggregates

Recently, Kato *et al.* [46] reported the copurification of αB-crystallin and the human small HSP from pectoral muscle. Zantema *et al.* [40] were able to immunoprecipitate both αB-crystallin and the mouse small HSP from non-oncogenic adenovirus-transformed cell lysates, using antibodies directed against either of the two proteins. These results strongly suggest the *in vivo* occurrence of αB-crystallin and small HSPs in the same aggregate. The questions arise whether mixed aggregates of αB-crystallin and HSP25 can

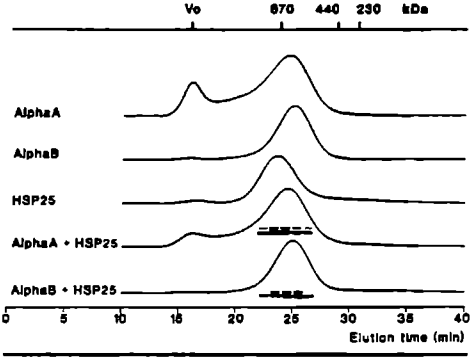


Fig. 3: Gel permeation analysis of αA-crystallin, αB-crystallin and HSP25, and of αA/HSP25 and αB/HSP25 mixtures. Superose 6B chromatography patterns of the indicated polypeptides and polypeptide mixtures, after denaturation and reaggregation. SDS-PAGE patterns of gel filtration fractions of the polypeptide mixtures are displayed directly under the corresponding elution profiles, the upper band represents HSP25, whereas the lower band represents αA or αB; lanes correspond with the fractions directly above them in the elution pattern. The void volume (V_o) and the position of gel filtration markers are indicated. High molecular weight aggregates, eluting at V_o, were sometimes observed.

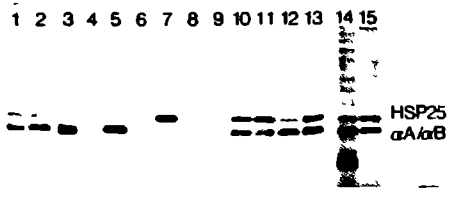


Fig. 4: Streptavidin blot of immunoprecipitated fractions of biotinylated αA-crystallin, αB-crystallin and HSP25, and of αA/HSP25 and αB/HSP25 mixed aggregates. Biotinylated αA/HSP25 (lane 1) and αB/HSP25 (lane 2) mixed aggregates were immunoprecipitated with anti-αA (lane 10) and anti-HSP25 (lane 11), or anti-αB (lane 12) and anti-HSP25 (lane 13), respectively. Controls are αA-crystallin, immunoprecipitated with anti-αA (lane 3) and anti-HSP25 (lane 4); αB-crystallin, immunoprecipitated with anti-αB (lane 5) and anti-HSP25 (lane 6); and HSP25, immunoprecipitated with anti-HSP25 (lane 7), anti-αA (lane 8) and anti-αB (lane 9). To demonstrate the selectivity of the aggregate formation, the water-soluble fraction of an HSP25-expressing *E.coli* strain was mixed with αB-crystallin (lane 14) and immunoprecipitated with anti-HSP25 (lane 15). The extra band in lane 7 represents a degradation product of HSP25.

also be assembled *in vitro*, and whether αA-crystallin/HSP25 mixed aggregates can be formed too.

To that end HSP25 was mixed with either αA- or αB-crystallin under denaturing conditions and reaggregated by dialysis against water. As a control, the pure polypeptides (αA, αB and HSP25) were subjected to the same reaggregation procedure. Dissociation of α-crystallin aggregates is known to be reversible [42, 47, 48] and the applied aggregation procedure has been shown to yield normal α-crystallin aggregates [e.g. 42]. The reaggregated polypeptides and their mixtures were analysed by gel permeation chromatography, at least in duplicate, resulting in elution volumes with variations less than 2%. From Fig. 3 it can be concluded that in all cases large aggregates are formed, although some minor differences in the elution volumes are observed. However, the elution volumes of αA-crystallin (12.28 ml) and the αA/HSP25 mixture (12.24 ml) correspond very well. The same is true for αB-crystallin (12.54 ml) and the αB/HSP25 mixture (12.52 ml). In the insets in Fig. 3 the SDS-PAGE compositions of the fractions corresponding with the peak of the elution pattern of the two mixtures are shown. Clearly, the polypeptides present in a mixture are coeluting.

It might well be that the proteins present in a mixture form separate homopolymers, that elute at the same volume. To rule out this possibility, an immunoprecipitation experiment was carried out. Biotinylation of the reaggregated polypeptides and their mixtures was performed to facilitate the detection of immunoprecipitated proteins by subsequent streptavidin blot analysis. The biotinylated polypeptide mixtures were treated with antisera, directed against only one of the two components of the mixtures, as described in Materials and Methods. The mixtures and their immunoprecipitation pellets were analysed by SDS-PAGE and streptavidin blot analysis (Fig 4). From lanes 10-13 it can be concluded that both components present in a mixture (αA and HSP25, or αB and HSP25) are precipitated by an antiserum specific for only one of the components. Since αA- and αB-crystallin show sequence homology with the small HSPs, it cannot *a priori* be excluded that the antisera raised against these polypeptides would cross-react. Therefore, controls for possible cross-reactivity were carried out (Fig. 4, lanes 3-9). In none of the cases an antiserum was able to precipitate the other polypeptide. Only in lane 6, where αB-crystallin was incubated with anti-HSP25 agarose beads, a very weak αB-crystallin band was observed. However, this weak signal cannot explain the strong signal obtained in lane 13. Therefore, it is more

likely that the weak band in lane 6 is caused by insufficient washing of the immuno-precipitation pellet. By comparing lanes 10 and 12 with lanes 11 and 13, respectively, it can be seen that the anti-HSP25 antibody precipitates relatively more HSP25 from the mixed aggregates than do the anti-αA or anti-αB antibodies. This implies that not all HSP25 subunits are necessarily present within the mixed aggregates. To demonstrate the selectivity of the aggregate formation between α-crystallin and HSP25, the water-soluble fraction of an HSP25-expressing *E.coli* strain, in which most bacterial proteins are present, was mixed with αB-crystallin and treated like the other protein mixtures. From all proteins present, only αB and HSP25 were immunoprecipitated with the anti-HSP25 antiserum (Fig. 4, lanes 14, 15). These experiments demonstrate that mixed aggregates of αA/HSP25 and αB/HSP25 can be formed *in vitro*. This strongly suggests that the subunit positions in the quaternary structure can be equally well occupied by αA, αB or HSP25.

Inhibition of thermal aggregation of β-crystallin by α-crystallin and HSP25

Recently, it has been shown that α-crystallin and its isolated subunits function as molecular chaperones, protecting proteins against heat-induced aggregation [31]. Since a molecular chaperone-like function would be attractive for the small HSPs too, we repeated the experiment with the mouse HSP25. To this end, a solution of ßL-crystallin was heated to 58°C in the absence or presence of HSP25 or α-crystallin. Apparently, the heat-induced aggregation of ßL-crystallin (Fig. 5, curve 1) is suppressed by HSP25 and α-crystallin (Fig. 5, curves 3 and 4). Assuming the average native molecular weight of ßL to be ~60,000 and for HSP25 to be around 600,000, a molar ratio of about 40:1 of ßL to HSP25 produces a significant effect in delaying the aggregation process and in lowering the rate of aggregate formation (Fig. 5, curve 2). With a molar ratio of 20:1 of ßL to HSP25, aggregation is almost completely suppressed (Fig. 5, curve 3). Additionally, the HSP25-induced heat-protection of yeast α-glucosidase at 49°C was tested. Similar results were obtained, with a molar ratio of 10:1 of α-glucosidase to HSP25, resulting in complete suppression of aggregation (results not shown).

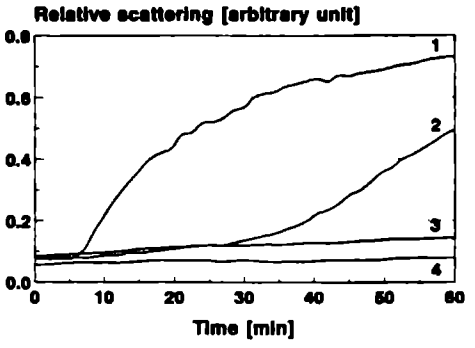


Fig. 5: Aggregation of ßL-crystallin at 58°C in the absence and presence of HSP25 or α-crystallin. In each experiment 0.13 mg ßL in 50 mM sodium phosphate, pH 7, was used. Aggregation of ßL was measured, either alone (curve 1), or in the presence of 0.034 mg HSP25 (curve 2), 0.067 mg HSP25 (curve 3) or 0.075 mg α-crystallin (curve 4). The final volume in each experiment was 0.4 ml.

α-Crystallin and HSP25 as substrates for transglutaminase.

Tissue transglutaminase catalyses the formation of ε-(γ-glutamyl)-lysine isopeptide crosslinks between polypeptides, and the conjugation of amines to protein-bound glutamines [49, 50]. It has been shown [30] that αB-crystallin, but not αA-crystallin, can serve as an amine-donor substrate in the transglutaminase-mediated crosslinking. Bovine αB-crystallin differs from αA-crystallin in having two lysine residues at its C-terminus [51]. It is indeed the C-terminal lysine of αB-crystallin that has been

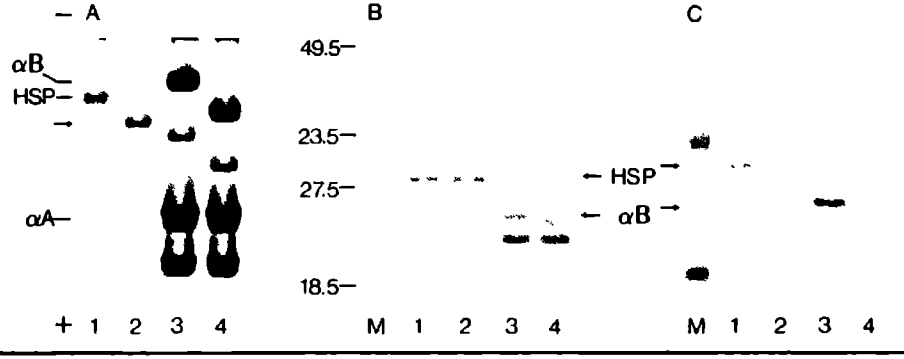


Fig. 6: Lysine-substrate capacity of α-crystallin and HSP25 in the transglutaminase mediated crosslinking. (A) Alkaline urea gel electrophoresis pattern of mouse HSP25 (1 μg, lanes 1 and 2) and bovine α-crystallin (20 μg, lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) carboxypeptidase B digestion. The distance between intact and truncated HSP25 (arrow) is approximately equal to the distance between intact and truncated αB-crystallin, demonstrating the loss of a single positive charge, due to the removal of the C-terminal lysine residue [30]. Carboxypeptidase B does not affect αA-crystallin. (B,C) SDS gel electrophoretic pattern after CBB-staining (B) or streptavidin blotting (C) of mouse HSP25 (lanes 1 and 2) and calf α-crystallin (lanes 3 and 4) before (lanes 1 and 3) and after (lanes 2 and 4) digestion with carboxypeptidase B. Note that the loss of a single lysine residue in αB-crystallin, but less so in HSP25, leads to a detectable higher mobility in SDS-PAGE. All samples have been incubated with Ca^{2+}-activated transglutaminase and the amine-acceptor probe. Comparison of the CBB pattern (B) and the corresponding streptavidin blot (C) shows that αB-crystallin and HSP25, but not αA-crystallin, are amine-donor substrates. Carboxypeptidase B digestion results in loss of the amine-donor capacity. Molecular masses of the markers in lane M are indicated in kDa.

identified as the amine-donor residue. Since also the mouse HSP25 contains a C-terminal lysine [33], the question arises whether this lysine residue can serve as an amine-donor as well. To address this question the purified recombinant mouse HSP25 and, as a control, α-crystallin were incubated with transglutaminase, Ca^{2+} and the biotinylated amine-acceptor probe, and analysed as described in Materials and Methods. Obviously, HSP25 can be decorated, like αB-crystallin, with the biotinylated hexapeptide (Fig. 6B,C, lanes 1 and 3), whereas αA-crystallin is not decorated (Fig. 6B,C, lane 3). This demonstrates that HSP25 can serve as amine-donor substrate. To identify the amine-donor residue, HSP25 and α-crystallin were treated with carboxypeptidase B, to remove the C-terminal lysine residue. The loss of a single positive charge, and thus the effectiveness of the digestion, is clearly demonstrated by alkaline urea gel electrophoresis (Fig. 6A). Subsequent reaction with the amine-acceptor probe, and analysis by SDS-PAGE and streptavidin blot shows that both HSP25 and αB-crystallin no longer react with the probe upon removal of the C-terminal lysine (Fig. 6B,C, lanes 2 and 4). This convincingly demonstrates that the C-terminal lysine in HSP25, as in αB-crystallin, serves as the amine-donor residue.

Inhibition of elastase

It has been shown that α-crystallins are efficient inhibitors of elastase [29]. It appears that α-crystallin inhibits elastase for 90% at stoichiometric levels, based on 800 kDa aggregates. We investigated whether also HSP25 abolishes the hydrolysis by elastase of a synthetic substrate as described in Materials and Methods. Fig. 7 demonstrates that indeed HSP25 inhibits

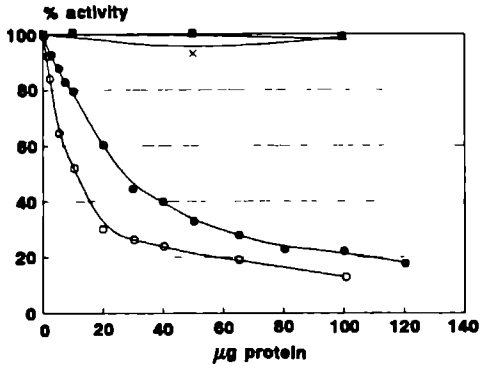


Fig. 7: Elastase inhibition curves of HSP25, α-crystallin and controls. Elastase activity was measured spectrophotometrically (Materials and Methods) after incubation with increasing amounts of HSP25 (●), α-crystallin (○), γ-crystallin (×) and ßH-crystallin (■). Data are presented as percentage of the activity of elastase alone.

elastase, although somewhat less pronounced than α-crystallin. To confirm that elastase inhibition by α-crystallin and HSP25 is specific, other bovine crystallins were tested, as well as bovine serum albumin, for their ability to inhibit elastase. In Fig. 7 the inhibition curves are included for γ- and ßH-crystallin, showing that these crystallins do not influence elastase activity. The same results were obtained for ßL-crystallin and bovine serum albumin, using up to 100 μg of these proteins (data not shown). Obviously, α-crystallin and HSP25 are efficient and specific inhibitors of elastase under the conditions used in this assay.

DISCUSSION

Among the ubiquitous family of small heat shock proteins, the α-crystallins are most closely related with the mammalian and avian 24-28 kDa HSPs [3]. The sequence homology between α-crystallins and the mammalian small HSPs [52, 53], the resemblance of their hydropathy profiles [53, 54], their similar aggregate size and electron microscopic appearance [19-27], all make it likely that they have the same conformations. α-Crystallin and small HSP subunits are likely to have a compact two-domain structure, with a short extending C-terminal arm [55]. Unfortunately, however, direct information about the spatial conformation of both α-crystallins and small HSPs is very limited. Having thus far resisted crystallization, no X-ray diffraction studies are available for these proteins. Similarly, the quaternary structure of α-crystallin is a matter of vigorous debate. Arguments have been presented for a three-layered spherical model [56, 57] and for a micellar structure [58], as well as for a combination of both [59]. For mouse HSP25 a sphere-like structure composed of about 32 monomers, arranged in hexagonal packing, has been proposed [45]. Direct measurements for secondary structure elements have only been published for α-crystallins, revealing primarily ß-sheet conformation and very little α-helical structure [41, 42, 47].

One of the aims of the present work is to provide additional evidence for the similarity of the secondary and quaternary structures of α-crystallin and small HSPs. The far UV CD spectra of bovine α-crystallin and mouse HSP25 clearly demonstrate that the secondary structure of HSP25 resembles very much that of α-crystallin, both consisting of mainly ß-pleated sheets. This finding supports the assumption that α-crystallin and HSP25 have similar conformations.

To provide evidence for similar structural stability of αA-crystallin, αB-crystallin and HSP25, the dissociation of the aggregates in urea was assessed by tryptophan fluorescence measurements. Normally, tryptophan fluorescence measurements are used to monitor tertiary structure trans-

itions, *i.e.* unfolding. For α-crystallin, however, it has been shown that the transition of fluorescence maxima occurring at 2.5 M is rather the result of a quaternary structure transition, *i.e.* dissociation [42, 44]. Therefore, we further consider the observed transitions for the α-crystallin subunits and HSP25 the result of dissociation. At pH 7.0 all three denaturation curves have equal transition points (2.5-2.6 M urea). At lower pH, αA-crystallin and HSP25 homopolymers remain equally stable, whereas the stability of αB-crystallin decreases significantly (1.5 M at pH 6.0). This seems in agreement with the observation of Augusteyn *et al.* [60] that αB-crystallin specifically dissociates from the α-crystallin aggregate at low pH. Apparently, this is due to destabilization of the quaternary structure interactions of αB-crystallin at lower pH. The fact that, at pH 7.0, all three proteins are equally stable, suggests that under physiological conditions similar interactions are playing a role in the aggregate formation of the three homopolymers. Also the fact that mixed aggregates can be formed from any combination of the αA-crystallin, αB-crystallin and HSP25 subunits implies that similar quaternary structures, with equivalent subunit positions in the aggregates, are formed.

Like their structural properties, the actual biological functions of both the small HSPs and α-crystallins are still largely unknown. In the lens, α-crystallin is assumed to have a structural role because of its very abundance [61]. However, the original function of αB-crystallin, and probably of αA-crystallin as well, must be looked for outside the lens. It is becoming increasingly clear that αB-crystallin and the small HSPs share important properties that are relevant for their common functioning as stress proteins (reviewed in [3]). These include stress-inducibility [9-11] and intracellular relocalization upon stress [9, 16, 18]. Both proteins appear to be involved in major cytomorphological reorganizations, both during normal development and under pathological conditions [13-16, 62-66]. Common features of α-crystallins and small HSPs are also their interactions with plasma membranes [67-70] and cytoskeletal elements [71-74], and the fact that they can be phosphorylated [34, 75-77]. To strengthen the notion that α-crystallin and the small HSPs indeed have common functions, we investigated whether calf α-crystallin and mouse HSP25 share some more properties. It has recently been found that αA- as well as αB-crystallin can act in several aspects like molecular chaperones [31]. α-Crystallin is able to prevent proteins from heat-denaturation and is helpful in refolding chemically denatured γ-crystallin. In this work we demonstrate that the mouse small HSP is able, like α-crystallin, to prevent heat-induced aggregation of βL-crystallin and of yeast α-glucosidase at substoichiometric levels. Comparable proof for the chaperone-like properties of HSP25 and α-crystallin has independently been obtained by Jakob *et al.* [78]. It is very likely that the stabilizing capacity of these proteins is of significance also *in vivo*, preventing undesirable protein interactions during periods of stress.

While the chaperone-like role is most probably the most prominent feature of α-crystallin and small HSPs, there are some other properties of potential functional importance that might be shared between these proteins. Recently, it has been shown that the C-terminal lysine of αB-crystallin is an amine-donor substrate in the transglutaminase-mediated crosslinking reaction of proteins [30]. We now demonstrate that HSP25 has the same substrate capacity and that, like in αB-crystallin, the C-terminal lysine is the amine-donor residue. This indicates that transglutaminase does not have very strict amine-donor substrate requirements, the C-terminal sequences of αB-crystallin and

HSP25 being -Ala-Val-Thr-Ala-Ala-Pro-Lys-Lys-OH and -Lys-Ser-Glu-Gln-Ser-Gly-Ala-Lys-OH, respectively [33, 51]. Our findings confirm in an indirect manner that the C-terminal lysines of αB-crystallin and HSP25, being accessible for transglutaminase, are located in extending arms. It is unclear what implications the substrate capacity for transglutaminase may have for αB-crystallin and HSP25 under normal conditions. It has been proposed that transglutaminase activity is implicated in controlled cell death [79], tumor progression [80], stimulated secretion [81], and receptor-mediated endocytosis [82]. These are processes which involve changes in the intracellular organization. The fact that αB-crystallin and small HSPs are also implicated in cytomorphological changes may provide a clue for the interactions between these proteins and transglutaminase.

α-Crystallin is known to inhibit elastase activity [29]. This property may be of functional importance in the lens. Because there is no protein turnover in the lens, the prevention of untimely degradation of lens proteins by endogenous proteases is of great importance. A similar protease inhibiting capacity might be useful for small HSPs as well, to avoid the proteolysis of transiently unfolded proteins during stress. By incubating elastase with increasing amounts of HSP25, we could demonstrate that also HSP25 is able to inhibit elastase activity, albeit somewhat less efficiently than α-crystallin. This may be due to improper folding of HSP25, or to differences in aggregate size. It should be noted that the elastase inhibition experiments are carried out in a low ionic strength buffer, not corresponding with the actual conditions in lens or other tissues. Under conditions with higher ionic strength, α-crystallin is not able to inhibit elastase (results not shown). However, elastase is most probably not the actual serine protease that is inhibited *in vivo* by α-crystallin and HSP25. Other, yet unknown proteases might be efficiently inhibited under more physiological conditions.

The original biological role of both αA- and αB-crystallin is most probably, like for the small HSPs, to function as stress proteins in tissues outside the lens. Their thermostability [83] and chaperone properties make it understandable that the constitutively high expression of α-crystallin in the eye lens is an evolutionarily useful feature for maintaining lens transparency. They thus resemble the enzyme/crystallins, like lactate dehydrogenase B and α-enolase, which also have been recruited unaltered to function as abundant structural proteins in the lenses of certain vertebrates [58].

REFERENCES

1. Ingolia, T. D., and Craig, E. A. (1982) Proc. Natl. Acad. Sci. USA 79, 2360-2364.
2. Van den Heuvel, R., Hendriks, W., Quax, W., and Bloemendal, H. (1985) J. Mol. Biol. 185, 273-284.
3. de Jong, W. W., Leunissen, J. A. M., and Voorter, C. E. M. (1992) Mol. Biol. Evol., 10, 103-126.
4. Bhat, S. P., and Nagineni, C. N. (1989) Biochem. Biophys. Res. Commun. 158, 319-325.
5. Dubin, R. A., Wawrousek, E. F., and Piatigorsky, J. (1989) Mol. Cell. Biol. 9, 1083-1091.
6. Iwaki T., Kume-Iwaki A., and Goldman, J. E. (1990) J. Histochem. and Cytochem. 38, 31-39.
7. Kato, K., Shinohara, H., Kurobe, N., Inaguma, Y., Shimizu, K., and Ohshima, K. (1991) Biochim. Biophys. Acta 1074, 201-208.
8. Kato, K., Shinohara, H., Kurobe, N., Goto, S., Inaguma, Y., and Ohshima, K. (1991) Biochim. Biophys. Acta 1080, 173-180.
9. Klemenz, R., Fröhli, E., Steiger, R., Schäfer, R., and Aoyama, A. (1991) Proc. Natl. Acad. Sci. USA 88, 3652-3656.
10. Dasgupta, S., Hohman, T. C., and Carper, D. (1992) Exp. Eye Res. 54, 461-470.
11. Inaguma, Y., Shinohara, H., Goto, S., and Kato K. (1992) Biochem. Biophys. Res. Comm. 182, 844-850.

12. Iwaki, T., Kume-Iwaki, A., Liem, R. K., and Goldman, J. E. (1989) Cell 57, 71-78.
13. Duguid, J. R., Rohwer, R. G., and Seed, B. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5738-5742.
14. Lowe, J., McDermott, H., Pike, I., Spendlove, I., Landon, M., and Mayer, R. J. (1992) J. Pathol. 166, 61-68.
15. Iwaki, T., Wisniewski, T., Iwaki, A., Corbin, E., Tomokane, N., Tateishi, J., and Goldman, J. E. (1992) Am. J. Pathol. 140, 345-356.
16. Chiesi, M., Longoni, S., and Limbruno, U. (1990) Mol. Cell. Biochem. 97, 29-136.
17. Renkawek, K., de Jong, W. W., Merck, K. B., Frenken, C. W. G. M., van Workum, F. P. A., and Bosman, G. J. C. G. M. (1992) Acta Neuropathol. 83, 324-327.
18. Voorter, C. E. M., Wintjes, L., Bloemendal, H., and de Jong, W. W. (1992) FEBS Lett., 309, 111-114.
19. Collier, N. C., Heuser, J., Levy, M. A., and Schlesinger, M. J. (1988) J. Cell Biol. 106, 1131-1139.
20. Hockertz, M. K., Clark-Lewis, I., and Candido, E. P. M. (1991) FEBS Lett. 280, 375-378.
21. Arrigo, A. P., and Pauli, D. (1988) Exp. Cell Res. 175, 169-183.
22. Spector, A., Li, L.-K., Augusteyn, R. C., Schneider, A., and Freund, T. (1971) Biochem. J. 124, 337-343.
23. Arrigo, A. P., and Welch, W. J. (1987) J. Biol. Chem. 262, 15359-15369.
24. Nover, L., Sharf, K. D., and Neumann, D. (1983) Mol. Cell. Biol. 3, 1648-1655.
25. Longoni, S., Lattonen, S., Bullock, G., and Chiesi, M. (1990), Mol. Cell. Biochem. 97, 121-128.
26. Siezen, R., Bindels, J. G., and Hoenders, H. J. (1979) Exp. Eye Res. 28, 551-567.
27. Koretz, J.F., and Augusteyn, R. C. (1988) Curr. Eye Res. 7, 25-30.
28. Arrigo, A., Suhan, J., and Welch, W. J. (1988) Mol. Cell. Biol. 8, 5059-5071.
29. Ortwerth, B. J., and Olesen, P. R. (1992) Exp. Eye Res. 54, 103-111.
30. Groenen, P. J. T. A., Bloemendal, H., and de Jong, W. W. (1992) Eur. J. Biochem. 205, 671-674.
31. Horwitz, (1992) Proc. Natl. Acad. Sci. U.S.A., 89, 10449-10453..
32. de Jong, W. W., Zweers, A., Versteeg, M., and Nuy-Terwindt, E. C. (1984) Eur. J. Biochem. 141, 131-140.
33. Gaestel, M., Gross, B., Benndorf, R., Strauss, M., Schunk, W., Kraft, R., Otto, A., Bohm, H., Stahl, J., Drabsch, H., and Bielka, H. (1989) Eur. J. Biochem. 179, 209-213.
34. Gaestel, M., Schroder, W., Benndorf, R., Lippmann, C., Buchner, K., Hucho, F., Erdmann, V. A., and Bielka, H. (1991) J. Biol. Chem. 266, 14721-14724.
35. Merck, K. B., de Haard-Hoekman, W. A., Oude Essink, B. B., Bloemendal, H., and de Jong, W. W. (1992) Biochim. Biophys. Acta 1130, 267-276.
36. Pace, N. P., Shirley, B. A., and Thomson, J. A. (1990) *in* Protein Structure, a practical approach, ed. Creighton, T. E., IRL Press, Oxford.
37. Laemmli, U. K. (1970) Nature 227, 680-685.
38. Siezen, R. J., and Hoenders, H. J. (1979) Eur. J. Biochem. 96, 431-440.
39. Mulders, J. W. M., Hoekman, W. A., Bloemendal, H., and de Jong, W. W. (1987) Exp. Cell Res. 171, 296-305.
40. Zantema, A., Verlaan-de Vries, M., Maasdam, D., Bol, S., and van der Eb A. (1992) J. Biol. Chem. 267, 12936-12941.
41. Li, L.K., and Spector, A. (1974) Exp. Eye Res. 19, 49-57.
42. Siezen, R. J., and Bindels, J. G. (1982) Exp. Eye Res. 34, 969-983.
43. Manavalan, P., and Johnson, C. W. (1983) Nature 305, 831-832.
44. van den Oetelaar, P.J.M., and Hoenders, H.J. (1989) Biochim. Biophys. Acta 995, 91-96.
45. Behlke, J., Lutsch, G., Gaestel, M., and Bielka, H. (1991) FEBS Lett. 288, 119-122.
46. Kato, K., Shinohara, H., Goto,, S., Inaguma, Y., Morishita, R., and Asano, T. (1992) J. Biol. Chem. 267, 7718-7725.
47. Thomson, J. A., and Augusteyn, R. C. (1989) Biochim. Biophys. Acta. 994, 246-252.
48. Bloemendal, H., Bont, W. S., Jongkind, J. F., and Wisse, J. H. (1962) Exp. Eye Res. 1, 300-305.
49. Folk, J. E. (1983) Adv. Enzymol. 54, 1-56.
50. Lorand, L., and Conrad, S. (1984) Mol. Cell. Biochem. 58, 9-35.
51. van der Ouderaa, F. J., de Jong, W. W., Hilderink, A., and Bloemendal, H. (1974) Eur. J. Biochem. 49, 157-168.
52. Hickey, E., Brandon, S. E., Potter, R., Stein, G., Stein, J., and Weber, L. A. (1986) Nucleic Acids Res. 14, 4127-4145.
53. de Jong, W. W., Leunissen, J. A. M., Leenen, P. J. M., Zweers, A., and Versteeg, M. (1988) J. Biol. Chem. 263, 5141-5149.
54. Nagao, R. T., Czarnecka, E., Gurley, W. B.,

Schoeffl, F., and Key, J. L. (1985) Mol. Cell. Biol. 5, 3417-3428.
55. Wistow, G. (1985) FEBS Lett. 181, 1-6.
56. Bindels, J. G., Siezen, R. J., and Hoenders, H. J. (1979) Ophthalmic Res. 11, 441-452.
57. Tardieu, A., Laporte, D., Licinio, P., Krop, B., and Delaye, M. (1986) J. Mol. Biol 192, 711-724.
58. Wistow, G., and Piatigorsky, J. (1988) Science 236, 1544-1556.
59. Walsh, M. T., Sen A. C., and Chakrabarti, B. (1991) J. Biol. Chem. 266, 20079-20084.
60. Augusteyn, R. C., Ellerton H. D., Putilina, T., and Stevens A. (1988), Biochim. Biophys. Acta 957, 192-201.
61. Tardieu, A., and Delaye, M. (1988) Ann. Rev. Biophys. Chem. 17, 47-70.
62. Lee, Y. J., Hou, Z. Z., Curetty, L., and Corry, P. M. (1992) J. Cell. Physiol. 150, 441-446.
63. Scotting, P., McDermott, H., and Mayer, R J (1991) FEBS Lett. 285, 75-79.
64. Pauli, D., Tonka, C., Tissieres, A., and Arrigo, A. (1990) J. Cell Biol. 111, 817-828.
65. Gyorgyey, J., Gartner, A., Nemeth, K., Magyar, Z., Hirt, H., Heberlebors, E., and Dudits, D. (1991) Plant Mol. Biol. 16, 999-1007.
66. Iwaki, T., Iwaki, A., Liem, R. K. H., and Goldman, J. E. (1991) Kidney Int. 40, 52-56.
67. Mulders, J. W. M., Wojcik, E., Bloemendal, H., and de Jong, W. W. (1989) Exp. Eye Res. 49, 149-152.
68. Ifeanyi, F., and Takemoto, L. J. (1990) Curr. Eye Res. 9, 259-265.
69. Grimm, B., Ish-Shalom, D., Even, D., Glaczinski, H., Ottersbach, P., Kloppstech, K., and Ohad I. (1989) Eur. J. Biochem. 182, 539-546.
70. Adamska, I., and Kloppstech, K. (1991) Eur. J. Biochem. 198, 375-381.
71. Bloemendal, H., Berbers, G. A. M., de Jong, W. W., Ramaekers, F. C. S., Vermorken, A. J. M., Dunia, I., and Benedetti, E. L. (1984) *in* Human Cataract Formation, Ciba Fnd. Symposium 106, pp. 177-186, Pitman, London.
72. Fitzgerald, P. G., and Graham, D. (1991) Curr. Eye Res. 10, 417-436.
73. Maisel, H., and Perry, M. M. (1972) Exp. Eye Res. 14, 7-12.
74. Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M., and Geiger, B. (1991) J. Cell Biol. 114, 255-261.
75. Voorter, C. E. M., Mulders, J. W. M., Bloemendal, H., and de Jong, W. W. (1986) Eur. J. Biochem. 160, 203-210.
76. Chiesa, R., Gawinowicz-Kolks, M. A., Kleiman N. J., and Spector, A. (1987) Biochem. Biophys. Res. Comm. 144, 1340-1347.
77. Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., and Anderson, C. W. (1992) J. Biol. Chem. 267, 794-803.
78. Jakob, U., Gaestel, M., Engel,K. and Buchner, J. (1993) J. Biol. Chem. (In Press).
79. Fesus, L., Thomazy, V., and Falus, A. (1987) FEBS Lett. 224, 104-108.
80. Knight, C. R. L., Rees, R. C., and Griffin, M. (1991) Biochim. Biophys. Acta 1096, 312-318.
81. Bungay, P. J., Owen, R. A., Coutts, I. C., and Griffin, M. (1986) Biochem. J. 235, 296-278.
82. Davies, P. J. R., Davies, D. R., Levitzki, A., Maxfield, F. R., Milhaud, P., Willingham, M. C., and Pastan, I. H. (1980) Nature 283, 162-167.
83. Maiti, M., Kono, M., and Chakrabarti, B. (1988) FEBS Lett. 236, 109-114.

CHAPTER 3

Expression and aggregation of recombinant αA-crystallin and its two domains

Expression and aggregation of recombinant αA-crystallin and its two domains.

The 20-kDa αA and αB subunits of α-crystallin from mammalian eye lenses form large aggregates with an average molecular weight of 800,000. To get insight into the interactions responsible for aggregate formation, we expressed in *E.coli* the putative N- and C-terminal domains of αA-crystallin, as well as the intact αA-crystallin chain. The proteins are expressed in a stable form and in relatively high amounts (20-60% of total protein). Recombinant αA-crystallin and the C-terminal domain are expressed in a water-soluble form. Recombinant αA-crystallin forms aggregates comparable with α-crystallin aggregates from calf lenses, whereas the C-terminal domain forms dimers or tetramers. The N-terminal domain is expressed in an initially water-insoluble form. After solubilization, denaturation and reaggregation the N-terminal domain exists in a high-molecular weight multimeric form. These observations suggest that the interactions leading to aggregation of αA-crystallin subunits are mainly located in the N-terminal half of the chain.

INTRODUCTION

Until recently α-crystallin was known as a typical organ-specific structural protein of the vertebrate eye lens [for reviews see 1-3]. It is normally isolated in the form of large water-soluble aggregates (500-900 kDa), composed of two types of homologous subunits, αA and αB. The α-crystallins are structurally related with the ubiquitous small heat shock proteins (HSPs)[1] [4-6]. It has recently been established that both subunits also occur outside the lens [7-9]. The properties of αB in non-lens cells resemble those of the small HSPs [10].

Unfortunately, very little is known about the three-dimensional structure of both α-crystallin and the small HSPs. This seriously hampers the understanding of the biological functioning of these proteins. Like α-crystallin, the small HSPs form large aggregates (200-800 kDa) [11-14], and both proteins appear in the electron microscope as 10-17 nm globular, sometimes torus-like or hollow-core particles [12, 15-18]. The quaternary structure of α-crystallin is a matter of debate; although a three-layered structure seems to be the most favoured [19,20], a micelle-like model has also been propagated [21].

It has been proposed that the subunits of α-crystallin and small HSPs are composed of two similarly folded domains and a C-terminal tail [22]. A two-domain structure is supported by folding/unfolding studies of α-crystallin subunits [23]. Because the sequence homology between α-crystallins and the small HSPs is most pronounced in the C-terminal domains, it was suggested that the common tendency to aggregate derives from this part of the protein [4]. On the other hand, the N-terminal domain of the α-crystallin subunits is definitely more hydrophobic than the C-terminal domain. Thus makes it more likely that the

[1] The abbreviations used are: HSP, heat shock protein; αA2D, residues 64-173 of rat αA-crystallin; αA1D, residues 1-63 of calf αA-crystallin; WSF, water-soluble fraction; USF, urea-soluble fraction; UIF, urea-insoluble fraction; SDS, sodium dodecyl sulfate, PAA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; LMW, low molecular weight; HMW, high molecular weight; IPTG, isopropylthio-ß-D-galactoside; PMSC, phenylmethylsulfonyl chloride; CBB, Coomassie Brilliant Blue; bp, base pair; ds, double-stranded.

N-terminal parts of the polypeptides are largely responsible for the aggregation behaviour, and has indeed led to the micelle-hypothesis for α-crystallin aggregates [21]. To distinguish between these opposing views, and to demonstrate that α-crystallin subunits are indeed composed of two independently folded domains, we decided to produce the putative domains of αA-crystallin by recombinant-DNA methods, and study their properties.

MATERIALS AND METHODS

Bacterial strains, plasmids and reagents

E.coli strains HMS174 [24] and JM105 [25] were used as recipients in the cloning experiments. For overproduction of recombinant proteins either *E.coli* strain B BL21(DE3) or B BL21(DE3).pLysS was used. Plasmids used in the cloning experiments were pET3b and pET8c [24]. Ampicillin, chloramphenicol, IPTG, chicken egg white lysozyme, DNAase I and protease inhibitors were obtained from Sigma. Restriction enzymes, T4 DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories (BRL). The crosslinking agent dimethyl suberimidate was obtained from Pierce Chemical Co. TPCK-treated trypsin was purchased from Worthington Biochemical Corporation. Oligonucleotides were synthesized using a Cyclone Plus DNA synthesizer from Milligen/Biosearch.

Plasmid constructions

The partial cDNA clone pRLαA-1 as described by Moormann *et al.* [26] was used to make the expression construct pET3bαA2D, expressing residues 64-173 of rat αA-crystallin (αA2D, Fig. 1C). The clone pBLαA-1 [27], containing the entire coding sequence of bovine αA-crystallin was used to make the expression constructs pET8cαA and pET8cαA1D, encoding bovine αA-crystallin (173 residues) and residues 1-63 of bovine αA-crystallin (αA1D), respectively (Fig. 1 A,B). The pET expression system of Studier *et al.* [24] was used to make all the expression constructs. Sequences coding for the above mentioned proteins were ligated in the different expression plasmids with the help of adaptors, as described in the legend of Fig. 1. All adaptors were unphosphorylated and were ligated using the procedure described by Haymerle *et al.* [28]. The ligation mixtures were used to transform *E.coli* HMS174 or JM105 and the desired expression constructs were isolated according to Sambrook *et al.* [29].

Expression and purification

For expression, the constructs pET8cαA and pET8cαA1D were transformed in the host *E.coli* BL21(DE3), while the construct pET3bαA2D was transformed in the same host, containing the plasmid pLysS, to facilitate cell lysis. Induction was performed by making the culture 0.5 mM in IPTG when the culture reached an OD600 of 0.6-1. Three hours after induction, cells from a 450 ml culture were harvested, resuspended in 20 ml of TEN-buffer (50 mM Tris (pH 7.5), 0.3 M NaCl, 0.5 mM EDTA, 0.02 μM PMSC), containing 1/1000 volume of protease inhibitor stock solution (2 mg/ml pepstatin, 0.1 M benzamidin-HCl, 1 mg/ml antipain, 1 mg/ml bacitracin, 5 mg/ml soybean trypsin inhibitor, 2 mg/ml aprotinin), like all other solutions used in this procedure, and lysed either by incubation with 1 mg/ml chicken egg white lysozyme during 30 min on ice or by freeze-thawing (pLysS strain). DNA was degraded by DNAase I (2 μg/ml) on ice, during 1 hr. By centrifugation (30 min at 15,000xg) the suspension was divided into a water-soluble fraction (WSF) and a pellet. The washed pellet was resuspended in 25 ml of cracking buffer (0.01 M sodium phos-

phate, pH 7.2, 1% of ß-mercaptoethanol, 6 M urea, 0.02 μM PMSC). The urea-soluble fraction (USF) was obtained by centrifugation (45 min at 15,000xg). The pellet was washed with 25 ml of cracking buffer and centrifuged again and the urea-insoluble fraction (UIF) was obtained. Proportional amounts of each fraction were loaded either on an SDS-polyacrylamide gel [30] or a Tricine-SDS-polyacrylamide gel [31]. αA2D was purified from pET3bαA2D WSF by antibody affinity chromatography. An affinity column, specifically retaining the C-terminal sequence of αA-crystallin was made by binding 1 ml ascites fluid of the monoclonal antiserum Cr.I-1 [32] to 1.75 ml of cyanogen bromide activated Sepharose 4B (Pharmacia) according to Stevens and Augusteyn [33]. The affinity column was loaded with up to 15 mg of protein, derived from the WSF of pET3bαA2D. Proteins bound through non-specific interactions were then eluted with 0.1 M glycine-HCl (pH 5.0). Specifically bound protein was eluted with 0.1 M glycine-HCl (pH 2.5). Fractions containing αA2D were collected, dialysed against water and lyophilized.

Characterization

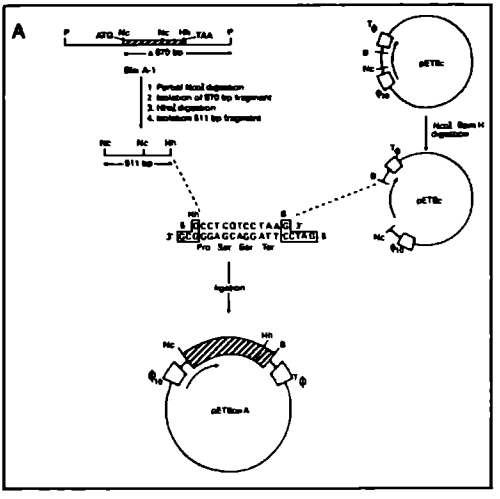
The apparent molecular weight of the expressed proteins was estimated by SDS-PAGE, while the immunochemical identity was established by Western blotting. A polyclonal antiserum raised against calf αA-crystallin was used to identify recombinant αA-crystallin. To identify αA2D, the monoclonal antiserum Cr.I-1 [32], reacting with C-terminal sequences of rat αA-crystallin, was used. For identification of αA1D, a polyclonal antiserum, directed against a synthetic peptide containing residues 49-63 of bovine αA-crystallin, was used [34].

Expressed proteins were further identified by analysis of their tryptic peptides. Tryptic digestion was performed on the WSF of recombinant αA-crystallin, containing up to 80% of the expressed protein. To obtain pure αA2D, the band containing αA2D was cut out from a SDS-polyacrylamide gel and isotachophoresed according to Öfverstedt *et al.* [35]. To obtain tryptic peptides of αA1D, the crude USF of αA1D was used. Tryptic peptides of the expressed proteins were separated either by high voltage paper electrophoresis at pH 6.5 and descending chromatography [36] or reversed phase HPLC on a RP-18 column (Merck) [37] and analysed, after hydrolysis, on a LKB-Alpha Plus amino acid analyser.

Aggregation

Aggregate sizes of recombinant proteins were estimated by means of FPLC gel permeation chromatography, using a Superose 6 HR 10/30 prepacked column (Pharmacia-LKB). The column was equilibrated with 50 mM phosphate (pH 7.5), 50 mM NaCl and 1 mM EDTA. The column was eluted at room temperature, at a flow rate of 0.5 ml/min and monitored by absorbance at 280 nm. Low and high molecular weight markers from the Pharmacia gel filtration calibration kit were used to estimate the molecular mass of the aggregates.

Samples (1 mg of protein) of the water-soluble fraction of bacteria expressing αA-crystallin or αA2D were directly applied to the gel filtration column. Additionally, affinity-purified αA2D was dissociated by 6 M urea in FPLC elution buffer, and reaggregated by dialysis against this phosphate buffer, the protein concentration being 0.2 mg/ml. αA1D was solubilized from the urea-insoluble pellet by dialysis against a buffer containing glycerol: 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSC, 10% glycerol. This dialysis step was simultaneously considered as a reaggregation step. After 2.5 fold concentration by ultrafiltration (Amicon 5),


A
pETBlc
pETBlcA
B
pETBlc
pETBlcA1D
C
pET28b
pET28bA2D

200 µl of the sample, containing 5 mg protein per ml, was applied to the gel filtration column.

Crosslinking

Crosslinking was performed according to Siezen *et al.* [38], with minor modifications. The WSF of an induced pET3bαA2D culture was dialysed overnight at 4°C against 0.2 M triethanolamine-HCl, 10 mM dithioerythritol, pH 8.0. Dimethyl suberimidate was dissolved in the same buffer and within 1 minute of preparation it was mixed with the protein solution, to final concentrations of 0.2-25 mM, final protein concentration of 1 mg/ml, and pH 8.0 at 20°C. The reactions were stopped after 2 h, by addition of SDS, up to a concentration of 1%, and immediate heating to 100°C for 2 min. To achieve a higher degree of crosslinking, protein solutions were treated with higher concentrations of dimethyl suberimidate (up to 125 mM) and a final incubation time of 6 h. All samples were analysed by Tricine SDS-PAGE.

RESULTS

Expression and purification of recombinant proteins

Proteins and protein fragments can be directly expressed as unfused polypeptides in *E.coli*, using the pET-expression system [24]. DNA fragments coding for bovine αA-crystallin (residues 1-173) and for its putative N-terminal domain, αA1D (residues 1-63), were inserted in the *Nco*I-site of the expression plasmid pET8c, resulting in the constructs pET8cαA and pET8cαA1D, respectively (Fig. 1A,B). The fragment encoding the C-terminal domain and tail, αA2D (residues 64-173), was inserted in the *Nde*I-site of plasmid pET3b, resulting in the expression construct pET3bαA2D (Fig. 1C).
The *E.coli* strain BL21(DE3) was used as

Fig. 1: Diagrams of construction of expression plasmids for αA-crystallin (A) and its N- and C-terminal domains (B and C, respectively). Hatched boxes indicate DNA sequences coding for bovine αA-crystallin (A), the N-terminal domain of bovine αA-crystallin (B) and the C-terminal domain of rat αA-crystallin (C). (A) The cDNA BLαA-1 (27) was partially digested with *Nco*I. The resulting ±870 bp fragment was further digested with *Hha*I. To insert the 511 bp *Nco*I-*Hha*I fragment of BLαA-1 into the expression vector pET8c, an unphosphorylated ds oligonucleotide was synthesized, containing the codons specifying the last three amino acids of bovine αA-crystallin and a TAA stop codon, as well as a *Bam*HI overhang site at the 3' end and an *Hha*I overhang site at the 5' end. Insertion of the BLαA-1 *Nco*I-*Hha*I fragment and the *Hha*I-*Bam*HI adaptor, resulted in the expression construct pET8cαA. (B) The *Nco*I-fragment of BLαA-1 encoding residues 1-107 of bovine αA-crystallin was digested with *Dde*I to obtain a 186 bp fragment. To ligate this *Nco*I-*Dde*I fragment into the expression vector pET8c, a synthetic unphosphorylated ds oligonucleotide was used containing the codon specifying residue 63 Glu of αA-crystallin, a TAG stop codon as well as a *Dde*I overhang site at the 5' end and an *Nde*I overhang site at the 3' end. The 3' *Nde*I overhang was present because this adaptor was originally designed for insertion of the *Nco*I-*Dde*I fragment into the *Nde*I site of the pET3b vector. The 5'-ends of the *Nco*I-*Dde*I fragment and the *Dde*I-*Nde*I adaptor were consecutively ligated to the vector and to the fragment. To circularize the construct the *Nde*I overhang site of the adaptor and the *Bam*HI overhang-site of the vector were filled in, using T4 DNA polymerase, the blunt ends phosphorylated and ligated. (C) The partial cDNA clone RLαA-1 (26) was digested with *Hpa*II. The resulting *Hpa*II fragment encodes residues 68-173 of rat αA-crystallin. To insert this fragment into the expression vector pET3b a synthetic unphosphorylated ds oligonucleotide was synthesized containing the ATG translation initiation codon, the codons specifying amino acids 64-67 of rat αA-crystallin, an *Nde*I overhang site at the 5' end and an *Hpa*II overhang site at the 3' end. The *Hpa*II fragment was ligated with both its 5' end and its 3' end to the *Nde*I overhang sites of the pET3b vector using the *Nde*I-*Hpa*II adaptor. P=*Pst*I, Nc=*Nco*I, Hh=*Hha*I, B=*Bam*H1, Dd=*Dde*I, Hp=*Hpa*II, Nd=*Nde*I.

the expression host for pET8cαA and pET8cαA1D, and their expression products were isolated as described in Materials and Methods. Induction of pET8cαA (Fig. 2A, lane 2) resulted in expression of a polypeptide with an apparent molecular weight a little smaller than the αA-crystallin subunit isolated from calf lenses (Fig. 2A, lane 6). This difference may be due to the N-terminal acetylation of calf lens αA-crystallin, which does not occur in *E.coli*. The identity of the induced protein was confirmed by Western blotting with a polyclonal anti-αA antiserum (Fig. 2A), and by amino acid analysis of tryptic peptides from the water-soluble bacterial lysate (data not shown). All expected peptides were present. The N-terminal peptide still contained the initiation methionine, as is the case in bovine αA-crystallin. This was expected, since it has been shown that methionine followed by an aspartic acid residue will not be removed in *E.coli* [39].

Recombinant αA-crystallin constitutes up

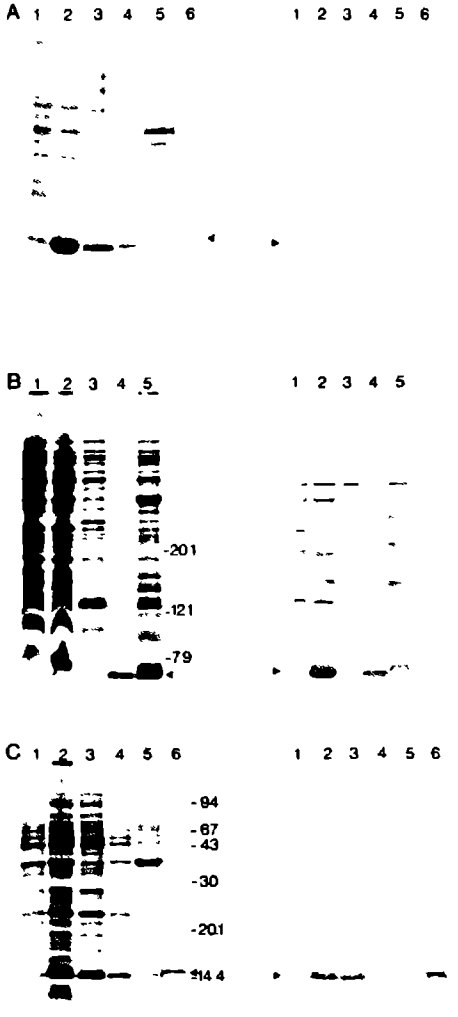


Fig. 2: Overexpression and extraction of αA-crystallin and its domains. Left halves of panels show CBB-stained protein patterns after SDS-PAGE, right halves of panels corresponding immunoblots. Molecular size markers, in kDa, are indicated. Arrowheads indicate the expressed proteins. (A) Lanes 1 and 2, total protein of *E.coli* BL21(DE3) containing plasmid pET8cαA, noninduced and induced, respectively. Lanes 3, 4 and 5, water-soluble, urea-soluble and urea-insoluble fraction, respectively, of the induced bacterial lysate. Lane 6, αA-crystallin isolated from calf lenses, as a control and a molecular weight marker. Samples were electrophoresed in a 13% SDS-PAA gel. The Western blot was reacted with a polyclonal antiserum, against calf αA-crystallin. (B) Lanes 1 and 2, total protein of *E.coli* BL21(DE3) containing plasmid pET8cαA1D, noninduced and induced, respectively. Lanes 3, 4 and 5, water-soluble, urea-soluble and urea-insoluble fraction, respectively, of the induced bacterial lysate. Samples were electrophoresed in a Tricine SDS-PAA gel. The Western blot was incubated with a polyclonal antiserum directed against residues 49-63 of αA-crystallin. (C) Lanes 1 and 2, total protein of *E.coli* BL21(DE3)pLysS containing plasmid pET3bαA2D, noninduced and induced, respectively. Lanes 3, 4 and 5, water-soluble, urea- soluble and urea-insoluble fraction, respectively, of the induced bacterial lysate. Lane 6, αA2D, purified by affinity chromatography. Samples were electrophoresed as in (B). For the Western blot, the monoclonal antiserum Cr.I-1, reacting with C-terminal sequences of rat αA-crystallin, was used.

to 60% of total bacterial protein, as estimated by gel scanning. The bacterial lysate was divided into a water-soluble fraction (WSF), a urea-soluble fraction (USF) and a urea-insoluble fraction (UIF). PAGE demonstrated that most of the recombinant αA-crystallin chain is in the WSF (Fig. 2A), however it contains only 40% recombinant αA-crystallin, as estimated by gel scanning. Since the WSF recovered from a 500 ml culture contains 80-150 mg protein, 30-50 mg αA-crystal-

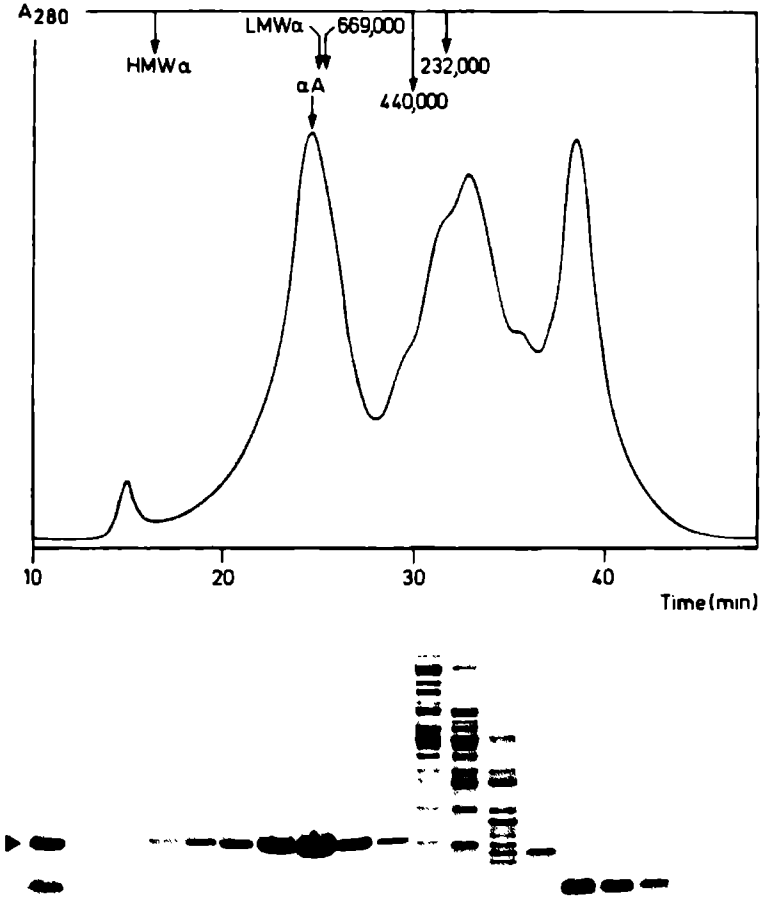


Fig. 3: Gel permeation analysis of recombinant αA-crystallin. (Top) Absorbance profile of the water-soluble fraction of induced *E.coli* BL21(DE3) containing plasmid pET8cαA, after Superose 6B chromatography. Elution positions of gel filtration markers as well as HMW and LMW bovine α-crystallin are indicated above the peaks. **(Bottom)** CBB-stained pattern after Tricine SDS-PAGE of alternate gel filtration fractions. Lanes correspond with the fractions directly above them in the elution pattern. The first lane represents the composition of the sample applied onto the gel filtration column. The arrowhead indicates the position of recombinant αA-crystallin.

lin is present in the WSF. Small amounts are in the USF and UIF, which typically is also the case for α-crystallin from bovine lenses [40].

Induction of pET8cαA1D resulted in the expression of a protein with an apparent molecular mass of about 7 kDa, which is the expected size for this oligopeptide (7.4 kDa) (Fig. 2B). αA1D accounts for about 40% of the total bacterial protein. The expressed protein is not recognized by polyclonal antibodies against αA-crystallin. We therefore used a polyclonal antiserum raised against residues 49-63 of calf αA-crystallin [34]. Unfortunately this antiserum cross-reacts with a number of bacterial proteins, since the immunized rabbit was contaminated with *E.coli*. However, a strong signal from a 7 kDa protein is only obtained after induction of the pET8cαA1D bearing strain. The identity of the expressed protein in the USF was confirmed by analysis of its tryptic peptides. All expected tryptic peptides were present, be it with contaminations due to the presence of other proteins in the USF. As can be seen from the molar ratio of the amino acid composition of T1: 1.4 Asp(1), 1.3 Glu(1), 1.0 Pro(1), 1.2 Ala(1), 0.8 Met(1), 1.7 Ile(2), 0.9 Phe(1), 0.8 His(1), 1.0 Lys(1) and Trp(1) (expected values [36] in brackets), the N-terminal methionine was not removed, like in recombinant αA-crystallin. The other peptides we found were: T2: Arg(1), T3: 1.0 Thr(1), 1.0 Ser(1), 2.0 Pro(2), 1.1 Gly(1), 1.1 Leu(1), 0.8 Tyr(1), 1.0 Phe(1), 1.0 Arg(1); T5: 0.8 Ser(1), 1.2 Glu(1), 1.0 Leu(1), 0.9 Phe(1), 1.1 Arg(1) and the expected part of T6: 1.2 Asp(1), 1.0 Thr(1), 1.7 Ser(2), 1.2 Glu(1), 1.1 Gly(1), 1.0 Val(1), 0.8 Ile(1), 1.0 Leu(1). The large and hydrophobic peptide T4 cannot be recovered by peptide mapping or RP-HPLC [36,37]. WSF, USF and UIF were also obtained for this strain. From Fig. 2B, lanes 3-5, it is clear that αA1D is not expressed as a water-soluble protein. The expressed protein is partially urea-soluble but considerable amounts remain in the urea-insoluble fraction. It may be that this protein is not properly folded, and as a result precipitates, co-precipitating a number of bacterial proteins (Fig. 2B, lane 5). To solubilize the proteins of the UIF, the UIF was dialysed against a glycerol-containing Tris-buffer (Materials and Methods). By this method almost all urea-insoluble proteins were dissolved, including αA1D. Once solubilized we could use αA1D for aggregation studies.

Chicken egg white lysozyme, used to lyse *E.coli* BL21(DE3) cells, has roughly the same molecular weight as αA2D. Therefore the strain *E.coli* BL21(DE3).plysS was used as the expression host for pET3bαA2D. This strain contains the plasmid pLysS, coding for T7-lysozyme, so lysis is simply performed by freeze-thawing [24]. Induction of the pET3bαA2D bearing strain led to the production of a protein with an apparent

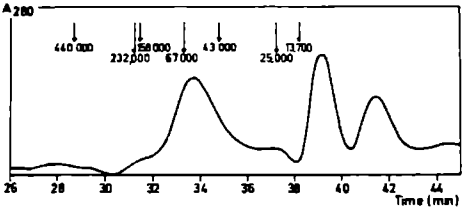


Fig. 4: Gel permeation analysis of affinity purified and reaggregated recombinant αA2D. Absorbance profile after Superose 6B chromatography of recombinant αA2D, purified by affinity chromatography from the water-soluble fraction of induced *E.coli*, containing plasmid pET3bαA2D, denatured in 6 M urea and reaggregated by dialysis. The first peak contains αA2D. The latter two peaks do not contain any protein, as was assessed by SDS-PAGE and amino acid analysis. Elution positions of gel filtration markers are indicated.

molecular mass of 14 kDa (Fig. 2C), which is slightly higher than the expected size of αA2D (12.4 kDa). αA2D accounts for about 20% of total cell protein. The expressed protein is recognized by the monoclonal antibody Cr.I-1, directed against an epitope near the C-terminus of rat αA-crystallin [32]. To further confirm the identity of the expressed protein, αA2D was isolated by isotachophoresis, after SDS-PAGE. Tryptic peptides of thus purified αA2D were analysed (data not shown). Indeed, all expected peptides were present. However, it turned out that, the initiation methionine was not efficiently removed in *E.coli*. This was not expected, in this case, since from the literature it appears that methionine with an adjacent valine should be removed for 90% [39]. Again WSF, USF and UIF were separated. Clearly, αA2D is expressed as a water-soluble protein (Fig. 2C, lanes 3-5). Moreover, αA2D is not degraded by bacterial proteases during expression in *E.coli*. These two facts indicate that a stable, properly folded structure has been formed [41]. αA2D was separated from bacterial proteins by affinity chromatography, as described in Materials and Methods (Fig. 2C, lane 6).

Aggregation behaviour of recombinant αA, αA1D and αA2D

To check whether native-like aggregates of recombinant αA-crystallin are formed during expression in *E.coli*, the crude WSF, containing recombinant αA, was applied onto a Superose 6B gel filtration column. From Fig. 3 it appears that the recombinant αA-crystallin indeed forms 700-800 kDa aggregates, that are well corresponding in size with the α-crystallin aggregates in the WSF of calf lenses [1]. This finding indicates that recombinant αA-crystallin is properly folded and aggregated in *E.coli*. Apparently, the absence of modifications, like N-terminal acetylation [36] and phosphorylation [37] does not influence the ability of forming aggregates of the proper size.

Prior to the determination of the molecular mass of αA2D, this protein was purified by affinity chromatography, denatured in 6 M urea and reaggregated as described in Materials and Methods. Pure reaggregated αA2D was applied to the Superose 6B gel filtration column. The elution profile in Fig. 4 shows three peaks of which the first contains αA2D, as established by SDS-PAGE. The first peak corresponds with a protein with an estimated molecular mass of 60 kDa, indicating that reaggregated αA2D is rather an oligomer than a polymer. When applying the crude WSF of

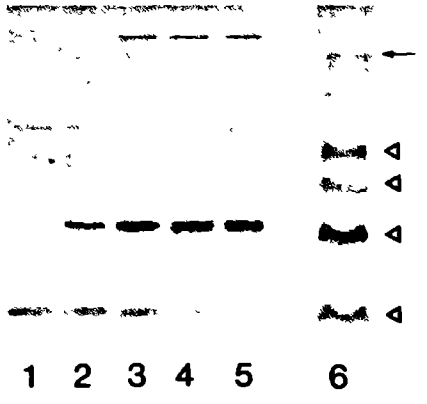


Fig. 5: Western blot of crosslinked recombinant αA2D. Crosslinking of bacterial WSF, at 1 mg/ml protein, for 2 h at 20°C, in 0.2 M triethanolamine-HCl (pH 8.0), 10 mM dithioerythritol, using 0 mM, 0.2 mM, 1 mM, 5 mM and 25 mM of dimethyl suberimidate (lanes 1-5, respectively). The sample in lane 6 was incubated for 6 h at a final concentration of 125 mM suberimidate, obtained by adding fresh aliquots of 25 mM each hour. A trace amount of dimeric αA2D is always present in the control non-crosslinked samples, despite the presence of ß-mercaptoethanol. Immunoblotting was performed with the monoclonal antibody Cr.I-1. Arrowheads indicate the positions of the mono-, di-, tri- and tetramers of αA2D. The arrow shows the boundary between stacking and resolving gel.

pET3bαA2D directly to the Superose 6B column the same result was obtained (data not shown). Gel permeation chromatography thus reveals that αA2D probably forms tetramers or pentamers. The quaternary structure was further analysed by cross-linking with dimethyl suberimidate (Fig. 5). The subunits of αA2D oligomers in the bacterial WSF were cross-linked at a total protein concentration of 1 mg/ml, to avoid interaggregate cross-linking. With increasing cross-linker concentrations, up

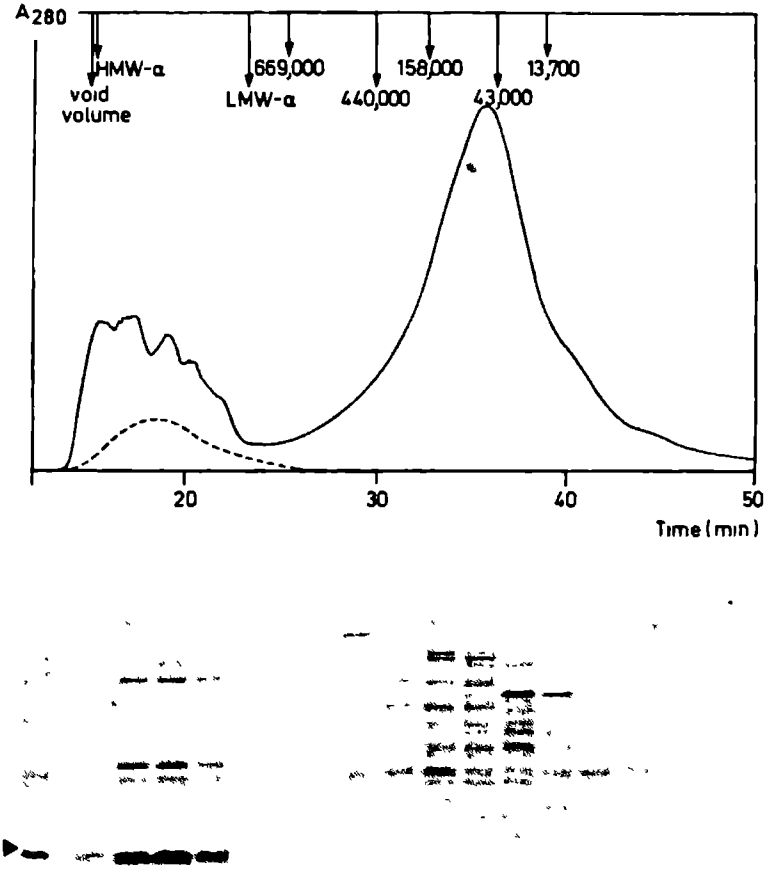


Fig. 6: Gel permeation analysis of recombinant αA1D. (Top) Absorbance profile of the solubilized urea insoluble fraction of induced *E.coli* BL21(DE3), containing plasmid pET8cαA1D, after Superose 6B chromatography. Elution positions of gel filtration markers as well as HMW and LMW α-crystallin are indicated above the peaks. The dashed line indicates the estimated presence of αA1D at an arbitrary scale. **(Bottom)** Pattern of alternate gel filtration fractions after Tricine SDS-PAGE. Lanes correspond with the fractions directly above them in the elution pattern. The first lane presents the composition of the sample applied to the gel filtration column. The arrowhead indicates the position of αA1D.

to 25 mM, αA2D forms predominantly dimers. Longer incubations, with repeated additions of a fresh portion of cross-linker, results in the formation of trimers and, clearly more strongly, tetramers. No greater multimers can be observed, except from some polymerized protein that is unable to penetrate the resolving gel. These results demonstrate that the αA2D oligomers occur as dimers or tetramers, the latter apparently consisting of a dimer of dimers.

To estimate the aggregate size of αA1D the urea-insoluble fraction was dialysed against a glycerol-containing Tris-buffer (Materials and Methods) to simultaneously solubilize and reaggregate αA1D. A sample containing 1 mg of protein was applied to the Superose 6B column. From Fig. 6 it appears that αA1D is able to form very large aggregates, ranging in size between HMW-α-crystallin (> 1,200 kDa) and LMW-α-crystallin (800 kDa). Alternatively, the USF of αA1D, in which the protein concentration (1 mg/ml) is considerably lower than in the solubilized UIF, was dialysed against the Superose 6B elution buffer, and analysed by gel filtration without prior ultrafiltration. Also in this case αA1D eluted after about 15 minutes, which corresponds well with the elution time found for αA1D from the solubilized UIF.

DISCUSSION

Recombinant αA-crystallin behaves like α-crystallin isolated from eye lenses, in terms of aggregation behaviour and solubility. Also, recombinant αA-crystallin is not susceptible to proteolytic cleavage during production in *E.coli*. This indicates that recombinant αA-crystallin has a stable conformation [41]. Apparently αA-crystallin is similarly folded and aggregated, whether it is expressed in *E.coli* or in lens cells. Unfortunately, not knowing any details of the biological functioning of αA-crystallin precluded the application of assays to establish the functional integrity of recombinant αA.

The fact that also αA2D is water-soluble and not susceptible to bacterial proteases again indicates that it is in a stable form, probably corresponding with its native conformation in the αA subunit. Urea denaturation and reaggregation of calf lens α-crystallin at low protein concentrations (<0.3 mg/ml) yields α-crystallin with an intact secondary and tertiary structure, as established by fluorescence and far ultraviolet circular dichroism measurements [42] and electron microscopy [19]. Urea denaturation and reaggregation of purified αA2D likewise yields oligomers with the same size as those formed directly in *E.coli*. It is, therefore, reasonable to assume that αA2D is properly folded. Its 'aggregation behaviour', i.e. the formation of dimers or tetramers, might thus be indicative for the interactions of this domain in its native environment, as part of the α-crystallin aggregate in the lens.

Unfortunately, reaggregation of αA1D at lower protein concentrations, to prevent superaggregation and precipitation, led to dramatic loss of αA1D during ultrafiltration steps. This may be caused by sticking of this hydrophobic polypeptide to the ultrafiltration membrane. However, aggregates obtained by reaggregation of relatively pure αA1D (the USF of Fig. 2B, lane 4), at a protein concentration of 1 mg/ml, have the same large size as aggregates formed during reaggregation of a protein mixture containing αA1D at higher protein concentrations. For α-crystallin it has been found that reaggregation of up to 50 mg/ml of pure α-crystallin results in aggregates that all have similar sizes [42]. It thus appears that, in contrast to αA2D, αA1D forms very large aggregates, under the conditions used. This is in accordance with earlier findings of Siezen *et al.* [17]. They observed that C-terminally truncated

αA subunits, obtained by partial tryptic digestion, generally tended to form larger aggregates the more of the C-terminus was cleaved off. The pronounced tendency of αA-crystallin to form large aggregates thus seems associated with the more hydrophobic N-terminal domain. This finding could be taken to fit the micellar model for α-crystallin as proposed by Augusteyn and Koretz [21]. Protein "micelles" might be formed through hydrophobic interactions between the N-terminal parts of the amphiphilic α-crystallin subunits. However, on basis of our data we can not exclude the possibility that αA1D is not an independent domain and that its aggregates are being formed through non-specific interactions, caused by inproper folding.

The aggregation properties of α-crystallin and the small HSPs are in all probability a crucial aspect of their biological functioning. It is known since long that α-crystallin in the eye lens forms larger aggregates (HMW-α) upon ageing and during cataractogenesis [1,2]. The aggregate size of α-crystallin is, among others, influenced by calcium ion concentration [43], temperature [44] and the presence of low molecular weight ß- and γ-crystallins [45]. In the rat heart αB-crystallin forms larger aggregates upon ischemia, a phenomenon that also takes place when the cytoplasmic fraction of control hearts is exposed to slightly acidic pH (6.5-7.0) [46]. The complexes of small HSPs likewise increase dramatically in size upon heat shock [11].

Our results agree with earlier suggestions of a two-domain structure for the α-crystallin subunits [22,23]. Further characterization of these now available domains of αA-crystallin, and of the corresponding domains of αB-crystallin and small HSPs, will contribute to solving the structure-function relationship of this intruiging family of proteins.

REFERENCES

1. Bloemendal, H. (ed.) (1981) Molecular and Cellular Biology of the Eye Lens, John Wiley and Sons, New York.
2. Harding, J.J., and Crabbe, M.J.C. (1984) in: The Eye (Davson, H., ed.), pp. 207-492, Academic Press, New York.
3. Wistow, G.J. and Piatigorsky, J., (1988) Annu. Rev. Biochem. 57, 479-504.
4. Ingolia, T.D., and Craig, E.A. (1982) Proc. Natl. Acad. Sci., U.S.A. 79, 2360-2364.
5. Lindquist, S., and Craig, E.A. (1988) Annu. Rev. Genet. 22, 631-677.
6. de Jong, W.W., Leunissen, J.A.M., Leenen, P.J.M., Zweers, A., and Versteeg, M. (1988) J. Biol. Chem. 263, 5141-5149.
7. Bhat, S. P., and Nagineni, C.N. (1989) Biochem. Biophys. Res. Commun. 158, 319-325.
8. Iwaki, T., Kume-Iwaki, A., and Goldman, J.E. (1990) J. Histochem. Cytochem. 38, 31-39.
9. Kato, K., Shinohara, H., Kurobe, N., Goto, S., Inaguma, Y. and Ohshima, K. (1991) Biochim. Biophys. Acta 1080, 173-180.
10. Klemenz, R., Fröhli, E., Steiger, R. H, Schäfer, R., and Aoyama, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3652-3656.
11. Arrigo, A., Suhan, J., and Welch, W.J. (1988) Mol. Cell. Biol. 8, 5059-5071.
12. Collier, N.C., Heuser, J., Levy, M.A., and Schlesinger, M.J. (1988) J. Cell Biol. 106, 1131-1139.
13. Hockertz, M.K., Clark-Lewis, I., and Candido, E.P.M. (1991) FEBS Lett. 280, 375-378.
14. Arrigo, A. P., and Paulı, D. (1988) Exp. Cell Res. 175, 169-183.
15. Nover, L., Sharf, K. D., and Neumann, D. (1983) Mol. Cell. Biol. 3, 1648-1655.
16. Longoni, S., Lattonen, S., Bullock, G., and Chiesi, M. (1990), Mol. Cell. Biochem. 97, 121-128.
17. Siezen, R., Bindels, J.G., and Hoenders, H.J. (1979) Exp. Eye Res. 28, 551-567.
18. Koretz, J.F., and Augusteyn, R.C. (1988), Curr. Eye Res. 7, 25-30.
19. Bindels, J.G., Siezen, R.J. and Hoenders, H.J. (1979) Ophthalmic Res. 11, 441-452.
20. Tardieu, A., Laporte, D., Licinio, P., Krop, B. and Delaye, M. (1986) J. Mol. Biol. 192, 711-724.

21. Augusteyn, R.C., and Koretz, J.F. (1987) FEBS Lett. 222, 1-5.
22. Wistow, G. (1985) FEBS Lett. 181, 1-6.
23. van den Oetelaar, P.J.M., and Hoenders, H.J. (1989) Biochim. Biophys. Acta 995, 91-96.
24. Studier, F. W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1989) Methods Enzymol. 185, 60-89.
25. Yanish-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103-119.
26. Moormann, R.J.M., van der Velden, H.M.W., Dodemont, H.J., Andreoli, P.M., Bloemendal, H., and Schoenmakers, J.G.G. (1981) Nucleic Acids Res. 14, 8615-8624.
27. Quax-Jeuken, Y., Quax, W., van Rens, G., Meera Khan, P., and Bloemendal, H. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 5819-5823.
28. Haymerle, H., Herz, J., Bressan, G.M., Frank, R., and Stanley, K.K. (1986) Nucleic Acids Res. 14, 8615-8624.
29. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Ed., pp. 1.25-1.28, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. Laemmli, U.K. (1970) Nature 227, 680-685.
31. Schägger, H., and von Gebhard, J. (1987) Anal. Biochem. 166, 368-379.
32. Hendriks, W., Sanders, J., de Ley, L., Ramaekers, F., Bloemendal, H., and de Jong, W.W. (1988) Eur. J. Biochem. 174, 133-137.
33. Stevens, A., and Augusteyn, R.C. (1988) Exp. Eye Res. 46, 499-505.
34. Takemoto, L., Emmons, T., Granstrom, D., and Jacobs, S. (1989) Biochim. Biophys. Acta 995, 259-263.
35. Öfverstedt, L.G., Sundelin, J. and Johansson, G. (1983) Anal. Biochem. 134, 361-367.
36. van der Ouderaa, F.J., de Jong, W.W., and Bloemendal, H. (1973) Eur. J. Biochem. 39, 207-222.
37. Voorter, C.E.M., Mulders, J.W.M., Bloemendal, H., and de Jong, W.W. (1986) Eur. J. Biochem. 160, 203-210.
38. Siezen, R.J., Bindels, J.G., and Hoenders, H.J. (1980) Eur. J. Biochem. 107, 243-249.
39. Dalbøge, H., Bayne, S., and Pedersen, J. (1990) FEBS Lett. 266, 1-3
40. Waley, S.G. (1969) in: The Eye (Davson, H., ed.), pp. 299-379, Academic Press, New York.
41. Parsell, D. A., and Sauer, R.T. (1989) J. Biol. Chem. 264, 7590-7595.
42. Thomson, J.A., and Augusteyn, R.C. (1984) J. Biol. Chem. 259, 4339-4345.
43. Jedziniak, J.A., Kinoshita, J.H., Yates, E.M., Hocker, L.O., and Benedek, B.G. (1972) Invest. Ophthalmol. 11, 905-915.
44. Thomson, J.A. and Augusteyn, R.C. (1983) Exp. Eye Res. 37, 367-377.
45. Mach, H., Trautman, P.A., Thomson, J.A., Lewis, R.V., and Middaugh, C.R. (1990) J. Biol. Chem. 9, 4844-4848.
46. Chiesi, M., Longoni, S., and Lumbruno, U. (1990) Mol. Cell. Biochem. 97, 129-136.

CHAPTER 4

Comparison of the homologous carboxy-terminal domain and tail of α-crystallin and small heat shock protein

Comparison of the homologous carboxy-terminal domain and tail of α-crystallin and small heat shock protein

The α-crystallins and small heat shock proteins (HSPs)[1] are structurally and functionally related. Especially the primary structure of their C-terminal domains is well-conserved. To study the significance of this feature, the C-terminal domain and tail of rat αA-crystallin and of mouse HSP25 were expressed in *E.coli*, and purified. The corresponding region of bovine αB-crystallin was obtained by cleavage of the protein with cyanogen bromide. Secondary structure analysis showed that all three domains have primarily β-sheet conformation and contain at most 10% of α-helix, like the proteins from which they are derived. Although the secondary structure thus appeared to be conserved in the three polypeptides, the quaternary structure was not. The C-terminal part of αA-crystallin has previously been shown to form dimers or tetramers, while the corresponding regions of αB-crystallin and mouse small HSP now appear to form larger aggregates. It has recently been shown that α-crystallin and small HSPs suppress heat-induced aggregation of proteins, and promote refolding of chemically denatured proteins. Although the C-terminal domains and tails of the three proteins apparently form viable structures, they did not show any heat protection activity. Finally, in the course of this study the primary structure of the C-terminal part of the mouse small heat shock protein was deduced from its cDNA sequence and confirmed by partial protein sequencing. Some differences with the previously published amino acid sequence were observed and a revision is therefore proposed.

INTRODUCTION

α-Crystallin, a major component of the vertebrate eye lens, forms large water-soluble aggregates (300-1000 kDa), composed of two types of homologous subunits, αA and αB (20 kDa) [1-3]. The α-crystallins are evolutionarily, structurally and functionally related with the ubiquitous small HSPs [4-6]. They are members of the α-crystallin/small HSP family [6,7]. Both α-crystallin subunits occur outside the lens. αA-Crystallin is found at very low levels in spleen and thymus [8], whereas αB-crystallin is expressed at higher levels, notably in heart, striated muscle and kidney [9-11]. Extralenticular αB-crystallin behaves as a small HSP [12]. Both α-crystallin and the small HSPs have been shown to have the properties of a molecular chaperone [13-15]. Both proteins form large aggregates, which is obviously related to their common functional features. However, no conclusive data about their quaternary structure is yet available. Different models for the arrangement of subunits in α-crystallin have been proposed, ranging from a multi-layer to a micellar arrangement of subunits [16-19]. A way to get more information about the quaternary structure is to find the regions that are involved in intersubunit interactions. The α-crystallin and small HSP subunits are probably composed of two compact structural domains and an extending C-terminal tail [20]. It has been

[1] The abbreviations used are: HSP(s), heat shock protein(s); HSP25, mouse small HSP; αA2Dt, residues 64-173 of rat α-crystallin; αB2Dt, residues 70-175 of bovine αB-crystallin; HSP2Dt, residues 92-209 of HSP25; bp, base pair; PMSF, phenylmethylsulfonyl chloride; WSF, water-soluble fraction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PAA, polyacrylamide; PCR, polymerase chain reaction; TCA, trichloroacetic acid.

proposed that the C-terminal domain, which is the best conserved sequence within this protein family, is the 'aggregation domain' [4]. We previously showed that the C-terminal domain and tail of αA-crystallin forms dimers or tetramers, whereas the N-terminal domain of αA-crystallin aggregates into less defined large complexes [21]. Based on this finding, a new model for the quaternary structure of α-crystallin and the small HSPs has been proposed, in which a rhombic dodecahedric arrangement of tetrameric building blocks occurs [22].
We now address the question as to whether also the corresponding C-terminal sequences of αB-crystallin and a small HSP form tetramers. Therefore we produced the C-terminal sequences of bovine αB-crystallin and mouse HSP25, and analysed their quaternary structures by gel permeation chromatography and crosslinking. Far UV circular dichroism spectroscopy was used to check the presence of regularly folded structure, and to find out whether the secondary structure of the C-terminal domains and tails is conserved. We further investigated whether the heat-protective capacity of the α-crystallin subunits and HSP25 is associated with the homologous C-terminal sequences.

MATERIALS AND METHODS

Bacterial strains, plasmids and reagents. *Escherichia coli* strains HB101 [23] and TG1 [24] were used as recipients for the constructs pAK3038p25 [25] and pET3bHSP2Dt, respectively. For overproduction of HSP2Dt, *E.coli* strain B BL21(DE3)pLysS [26] was used. The construction of pAK3038p25 has been described earlier [25], and pET3bαA2Dt

```
                * 10          20  *      30          40
HSP2Dt   MIRQTADRWRVSLDVNHFA---PEELTVKTKEGVVEITGKHEERQ
 αA2Dt   MVRSDRDKFVIFLDVKHFS---PEDLTVKVLEDFVEIHGKHNERQ
 αB2Dt     RLEKDRFSVNLDVKHFS---PEELKVKVLGDVIEVHGKHEERQ
                *                   *                 *

                *  50          60  *      70         *80
HSP2Dt          DEHGYISRCFTRKYTLPPGVDPTLVSSSLSPEGTLTVEA
 αA2Dt          DDHGYISREFHRRYRLPSNVDQSALSCSLSADGMLTFSG
 αB2Dt          DEHGFISREFHRKYRIPADVDPLAITSSLSSDGVLTVNG
                *                   *                 *

             90         100         110         120
HSP2Dt   PLPKAVTQS--AEITIPVTFESRAQIGGPEAGKSEQSGAK
 αA2Dt   PKVQSGLDAGHSERAIPVSREEKPSSAPSS
 αB2Dt   PRKQASG----PERTIPITREEKPAVTAAPKK
                                 ~~~~~~~~~~
```

Figure 1: Alignment of the homologous C-terminal domains and tails of the mouse small heat shock protein, rat αA-crystallin and bovine αB-crystallin [6]. The aligned sequences are displayed in three blocks, the first two corresponding with the two putative motifs in the C-terminal domain of α-crystallin, and the last one representing the C-terminal extension, as proposed by Wistow [20]. Lysine residues are printed in bold-type. The conserved aspartyl, prolyl and glycyl residues are indicated by an asterisk. At the bottom, putative interacting regions are represented. The very flexible C-terminal tail of αA- and αB-crystallin [37] is represented by the wavy line. The original sequence of HSP2Dt [25] was corrected as follows: 8(del→R), 18(V→F), 19(I→A) and 96(T→A), and are underlined.

has previously been prepared in our laboratory [21]. Ampicillin, chloramphenicol, IPTG, DNAase I, proteinase inhibitors and proteinase K were obtained from Sigma. Restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories (BRL), whereas Taq-polymerase was obtained from Perkin-Elmer. The cross-linking agent dimethyl suberimidate was from Pierce. Oligonucleotides were synthesized using a Cyclone Plus DNA synthesizer from Milligen/Biosearch.

Polymerase chain reaction and cloning procedures

Polymerase chain reactions were performed in a total volume of 50 μl, and contained 20 mM Tris/HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 mM dNTPs, 0.2 μM primers, ~10 pg of pAK3038p25 and 1.25 U of Taq-polymerase. After an initial incubation at 95°C for 8 min, 30 cycles were run. The first cycle consisted of 2 min at 95°C, 1 min at 51°C, and 2 min at 72°C. At each subsequent cycle, the elongation step was extended with 2 sec. After 30 cycli an extra incubation of 2 min. at 72°C was performed. The final reaction mixture was consecutively extracted with phenol/chloroform (1:1, v/v) and chloroform. It was then treated with proteinase K (0.5 mg/ml), extracted again with phenol/chloroform and chloroform, and stored at -20°C.

The DNA fragment (604 bp) obtained from the amplification reaction was digested with NdeI and BamHI and inserted in the expression plasmid pET3b. Plasmid DNA (pET3bHSP2Dt) was sequenced by the dideoxynucleotide method [27] and transformed in BL21(DE3)pLysS cells.

Expression and purification of proteins

αB-Crystallin was obtained from calf lenses as described [28]. Bovine βL-crystallin was purified by gel filtration [28]. Recombinant bovine αA-crystallin and mouse HSP25 were isolated according to Merck *et al.* [14] and Gaestel *et al.* [29], respectively. To be able to distinguish between the C-terminal domain with and without the extending tail, we will further refer to the former as 2Dt and to the latter as 2D. αA2Dt (previously αA2D) was expressed in *E.coli*. A water-soluble fraction of the *E.coli* lysate was obtained as described [21], dialysed against demineralized water, containing proteinase inhibitors (20 μM PMSF, 100 μM EDTA, 40μg/l bacitracin and 30 μg/l benzamidin) and lyophilized. 100 mg of lyophilized αA2Dt WSF was dissolved in starting buffer (1 mM sodium phosphate, pH 6.8), containing the above mentioned proteinase

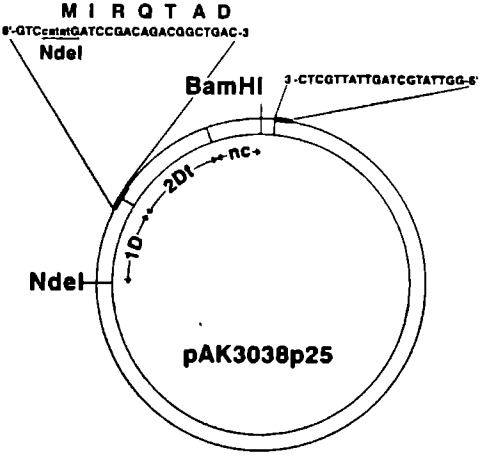


Figure 2: Construction of the HSP2Dt expression vector. The clone pAK3038, containing the full-length cDNA of mouse HSP25 [25] was used to amplify the fragment encoding HSP2Dt by the polymerase chain reaction. The resulting DNA fragment of 604 bp was cleaved with NdeI and BamHI and cloned in the NdeI/BamHI-cleaved pET3b vector to yield the pET3bHSP2Dt construct. 1D, 2Dt and nc are the nucleotide sequences corresponding with the N-terminal domain, C-terminal domain and tail, and the 3' non-coding region, respectively. Lower case letters in the N-terminal primer denote mismatches, to introduce an NdeI site.

inhibitors, and loaded on a Bio-Gel hydroxylapatite column (Bio-Rad), equilibrated with starting buffer. Proteins were eluted at a linear flow rate of 0.47 cm/min and with a linear gradient of sodium phosphate (1.4 mM/min). Fractions containing αA2Dt were pooled, dialysed against water (containing the above-mentioned proteinases) and lyophilized. Prepurified αA2Dt was dissolved in water, containing 0.1% TFA at the highest protein concentration possible (2-10 mg/ml). 1 ml of the dissolved sample was loaded on a reversed phase HPLC column (RP-4, Selectosil, 5C4, 300 Å) with dimensions of 250 mm x 4.6 mm. Proteins were eluted at a flow rate of 0.8 ml/min and with a gradient of acetonitrile in 0.1 % TFA. Fractions containing the desired polypeptides were pooled and lyophilized.
HSP2Dt was expressed using the bacterial strain BL21(DE3).pLysS containing the plasmid pET3bHSP2Dt. The expression of the construct and the preparation of water-soluble, urea-soluble and urea-insoluble fractions were performed as described previously [21]. HSP2Dt was directly purified from the WSF by RP-4 chromatography.
αB2Dt was obtained by cyanogen bromide cleavage of αB-crystallin according to standard procedures [30]. αB2Dt was isolated by RP-4 chromatography, as described above.

Reaggregation
Reaggregation was performed essentially as described earlier [14]. For crosslinking experiments, samples were dissociated in 6 M urea, diluted with 0.2 M triethanolamine-HCl (pH 8.0) to a final concentration of 1 M urea and 0.4 mg/ml of protein and dialysed against this buffer. For measuring heat protection activity and CD spectra the dissociated samples were diluted with water to a final concentration of 1 M urea and 1 mg/ml of protein, dialysed against water and lyophilized. All solutions used for reaggregation contained 50 mM of β-mercaptoethanol and proteinase inhibitors as indicated above.

Secondary structure estimation
Information about the secondary structure was obtained by far UV CD spectroscopy. CD measurements were carried out using a Jasco Model 600 spectropolarimeter. A 0.50 mm pathlength cell was used. Reaggregated proteins were dissolved in 50 mM sodium phosphate buffer, containing 0.1 M NaCl, pH 7.0. Protein concentrations were determined by amino acid analysis. The spectra, an average of 8 scans, are normalized to a concentration of 25 μM. The α-helix content was estimated according to Greenfield and Fasman [31].

Miscellaneous methods
The N-terminal amino acid sequence of HSP2Dt was determined on an Applied Biosystems Model 470A Protein Sequencer, equipped on-line with a Model 120A PTH Analyser. Gel permeation analysis was carried out, using a Superose 6 HR 10/30 prepacked column (Pharmacia-LKB) [21]. Samples containing approximately 25 μg of protein were applied to the column and absorbance was measured at 225 nm. To calibrate the gel filtration column, molecular mass markers from the Pharmacia gel filtration calibration kit were used. Crosslinking was performed with 25 mM of dimethyl suberimidate, as described earlier [13,21]. Crosslinking samples were analysed by Tricine SDS-PAGE [32] after TCA precipitation. The heat protection assay was performed as described earlier [14]. All samples used for the heat protection experiments were dissolved in 50 mM sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl. They were then centrifuged at 20,000 rpm for 20 min.

RESULTS

To compare the properties of the C-terminal domain with extending tail (abbreviated as 2Dt) of αA-crystallin, αB-crystallin and mouse HSP25 we could use previously described procedures to produce αA2Dt and αB2Dt, but had to construct first an expression plasmid for HSP2Dt. The respective sequences of the three proteins are aligned in Fig. 1.

Construction and sequence of pET3bHSP2Dt

Fig. 2 summarizes the strategy chosen for the creation of a clone capable of directing the synthesis of the C-terminal domain and tail of HSP25, by means of the polymerase chain reaction. The clone pAK3038p25, containing the full-length cDNA of mouse HSP25 [25] was used to amplify the desired fragment. This fragment starts at nucleotide 364 of the cDNA p25, corresponding with isoleucine-92 of mouse HSP25 [25], and comprises the 3' sequence of the p25 cDNA, including the entire 3' noncoding region, as well as 69 nucleotides downstream from the BamHI cloning site of pAK3038p25 [25]. The 5'-primer used in the reaction contained mismatches to introduce an NdeI restriction site and a translation initiation codon. The DNA fragment encoding HSP2Dt was cloned into the pET3b expression vector and transformed. To check whether or not mutations had occurred during the polymerase chain reaction, the insert was sequenced. Several differences with the expected sequence [25] were detected. The inferred differences corresponded with 3 amino acid substitutions and the insertion of one amino acid (see Fig. 1). Three of these differences were limited to the nucleotide sequence corresponding with the 22 N-terminal residues, so we resorted to N-terminal protein sequencing and confirmed that the amino acid sequence corresponded with the nucleotide sequence. The results of our protein and DNA sequencing are depicted in Fig. 1. Our corrected amino acid sequence of HSP25 better corresponds with that of chinese hamster HSP27 [33] and human HSP27 [34,35].

Purification of αA2Dt, αB2Dt and HSP2Dt

A prepurification of αA2Dt from the water-soluble fraction of an *E.coli* strain overexpressing αA2Dt [21] was performed by hydroxylapatite chromatography, resulting in about 80% pure αA2Dt (Fig. 3, lane 3). The elution profile of hydroxylapatite chromatography is shown in Fig. 4. By virtue of the fact that a single internal methionine residue is present at position

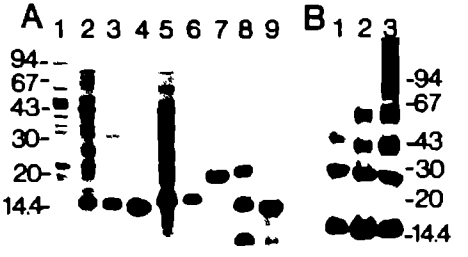


Fig. 3: Purification and crosslinking of αA2Dt, αB2Dt and HSP2Dt. (A) Tricine SDS-PAGE pattern of samples taken during the purification procedure of αA2Dt, αB2Dt and HSP2Dt. Lane 1, total protein of *E.coli* BL21(DE3).pLysS, containing plasmid pET3bαA2Dt without induction. Lane 2, WSF of this strain, after induction. Lane 3, αA2Dt after hydroxylapatite chromatography of the WSF of αA2Dt. Lane 4, αA2Dt after hydroxyl apatite and RP-4 chromatography. Lane 5, WSF of induced *E.coli* BL21(DE3).pLysS, containing plasmid pET3bHSP2Dt. Lane 6, HSP2Dt after RP-4 chromatography of the WSF of this strain. Lane 7, calf αB-crystallin. Lane 8, αB-crystallin after cleavage with cyanogen bromide. Lane 9, pure αB2Dt after RP-4 chromatography of cyanogen bromide cleaved αB-crystallin. (B) Tricine SDS-PAGE pattern of crosslinked samples of HSP2Dt (lane 1), αA2Dt (lane 2) and αB2Dt (lane 3). SDS-PAGE molecular mass markers are indicated in kDa.

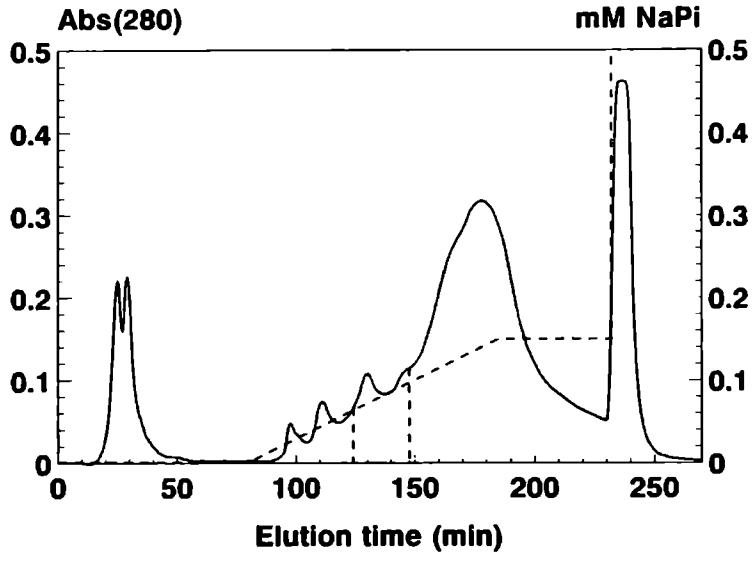


Fig. 4: Hydroxylapatite elution pattern of the *E.coli* water-soluble fraction containing αA2Dt. The solid line indicates the elution pattern, monitored by absorbance at 280 nm. The broken line indicates the sodium phosphate gradient that was applied to elute αA2Dt. Fractions corresponding with an elution time of 123-148 min (broken vertical lines) were collected, dialysed and lyophilized.

67 in bovine αB, precisely preceeding the C-terminal domain [6], αB2Dt could simply be obtained by cleavage with cyanogen bromide. This resulted in a mixture of three polypeptides: uncleaved αB-crystallin, residues 2-67 and residues 68-175 (αB2Dt) (Fig. 3, lane 8). HSP2Dt was expressed in *E.coli*, after transformation of the construct pET3bHSP2Dt in BL21(DE3)pLysS, reaching levels of up to 20 % of total protein (Fig. 3, lane 5). The *E.coli* lysate was fractionated in a water-soluble, urea-soluble and a urea-insoluble fraction. HSP2Dt turned out to be water-soluble.

The hydroxylapatite pool of αA2Dt, the CNBr cleavage mixture of αB-crystallin and the water-soluble fraction of the *E.coli* lysate containing HSP2Dt were applied to a reversed phase HPLC column of which typical elution patterns are shown in Fig. 5. Fractions containing the desired polypeptide were pooled and analysed by Tricine SDS-PAGE (Fig. 3). Although an analytical reversed phase column was used for the purification of the C-terminal domains, up to 10 mg of protein could be loaded on the column and a maximum of 3 mg of pure protein (αB2Dt) was obtained per run. Following the above procedures, a maximum of 10 mg of pure αA2Dt and HSP2Dt could be obtained from a 500 ml *E.coli* culture.

Quaternary structure of αA2Dt, αB2Dt and HSP2Dt

Previously we showed that αA2Dt forms dimers or tetramers [21]. To investigate whether also the conserved C-terminal sequence of αB-crystallin and HSP25 forms dimers or tetramers, we subjected the purified and reaggregated polypeptides to gel permeation chromatography (Fig. 6). In contrast with αA2Dt [21], both αB2Dt and HSP2Dt form large aggregates. On basis of gel permeation mass

markers, molecular masses were estimated (Fig. 6). From the calibration line, shown in Fig. 6, it is obvious that the calculation of molecular masses gives not more than a rough indication. αB2Dt and HSP2Dt form aggregates of approximately 235 kDa (18-20 subunits) and 170 kDa (12-13 subunits), respectively. However, the elution profile of αB2Dt is very asymmetric, indicating that αB2Dt is a heterogeneous population of aggregates, with the largest aggregate consisting of 18-20 subunits. To further analyse the quaternary structure, the polypeptides were crosslinked by 25 mM dimethyl suberimidate. In the case of αB2Dt more than 7 crosslinking products, all multiples of 15 kDa, can be distinguished (Fig. 3B). However, in the case of HSP2Dt, predominantly dimers are formed, and only a faint tetramer band can be observed. This seems to be in contrast with the results obtained with the gel permeation experiment. A possible explanation for this discrepancy is that the lysines of HSP2Dt are accessible to the crosslinker in a different manner than those of αB2Dt. It does, however indicate that HSP2Dt, like αA2Dt, occurs in a dimeric or tetrameric arrangement.

Secondary structure of αA2Dt, αB2Dt and HSP2Dt

All three domains are perfectly water-soluble, indicating that no improper interactions between secondary structure elements, leading to aspecific aggregation, take place. Far UV CD measurements were performed, in order to provide additional evidence that the domains are properly folded, and that they contain the same secondary structure elements as α-crystallin and HSP25, which are known to have predominantly β-sheet structure and at most 10% of α-helix. Prior to CD spectroscopy, all investigated proteins were denatured and refolded under the

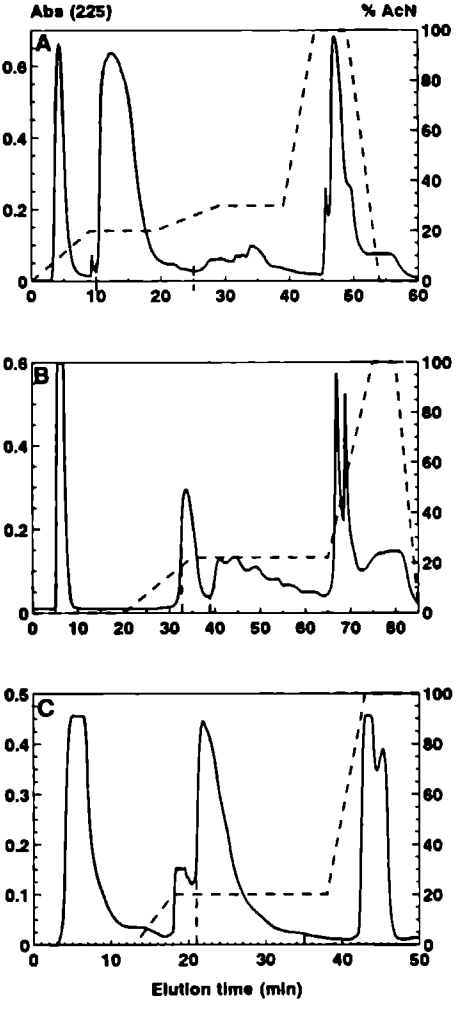


Fig. 5: Elution patterns of reversed phase chromatography of αA2Dt, αB2Dt and HSP2Dt. The solid lines indicate elution patterns of the hydroxylapatite pool of αA2Dt (A), the cyanogen bromide cleavage mixture of αB-crystallin (B) and the WSF of the *E.coli* strain overexpressing HSP2Dt (C), chromatographed on a Selectosil RP-4 column. Absorbance was monitored at 225 nm. The broken lines indicate the gradient of acetonitrile (in %), used to elute the polypeptides. Vertical broken lines indicate the elution time range in which the desired polypeptides eluted. Corresponding fractions were collected, lyophilized and analysed by Tricine SDS-PAGE (Fig. 3).

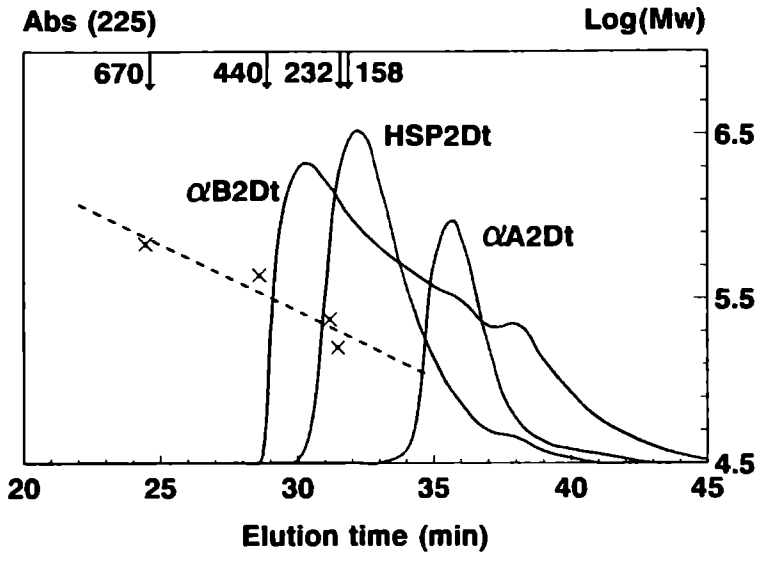


Figure 6: Gel permeation chromatography of αA2Dt, αB2Dt and HSP2Dt. Elution profiles of αB2Dt and HSP2Dt after Superose 6B chromatography, compared with that of αA2Dt. Absorbance was monitored at 225 nm. Elution positions of gel filtration markers are indicated at the top of the figure. Molecular masses are given in kDa. The broken line indicates the calibration line (elution time vs. log(Mw)).

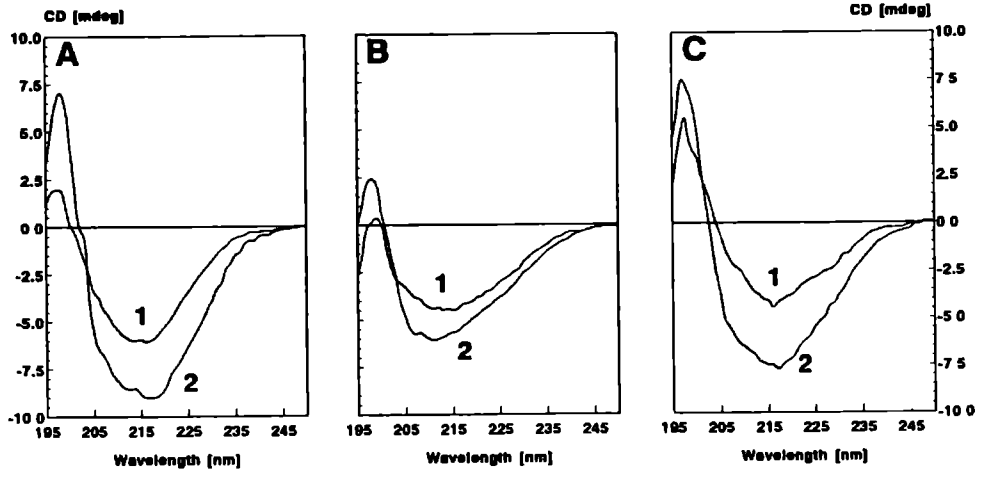


Figure 7: Far UV CD spectra of αA2Dt, αB2Dt and HSP2Dt. Panel A compares the CD spectra of αA2Dt (curve 1) and recombinant αA-crystallin (curve 2); panel B shows the CD spectra of αB2Dt (curve 1) and calf αB-crystallin (curve 2); panel C compares the CD spectra of HSP2Dt (curve 1) and recombinant HSP25 (curve 2). Cuvet path length is 0.5 mm in all cases. Spectra are normalized to a concentration of 25 μM.

same conditions. In Fig. 7, the normalized far UV CD spectra are shown. All three domains have curves that strongly resemble those of the proteins from which they are derived. The spectra differ in intensity, which is due to differences in molecular mass. It turns out that all proteins and protein fragments contain at most 10% of α-helix. This convincingly shows that, although the domains have different tendencies for oligomerization, their secondary structures are similar and intact. Moreover, the secondary structures of the C-terminal domains resemble each other, like is also the case for the α-crystallin subunits and HSP25.

Heat protection capacity of αA2Dt, αB2Dt and HSP2Dt

It has recently been discovered that both α-crystallin and HSP25 can function as molecular chaperones [13-15]. To investigate whether this function can be assigned to the C-terminal domain and tail of these proteins, a heat protection experiment was carried out. βL-crystallin was heated in the presence of the C-terminal sequences of αA, αB and HSP25. Heat denaturation of βL-crystallin was monitored spectrophotometrically. The heat denaturation curves are shown in Fig. 8. Obviously, none of the domains is capable of protecting βL-crystallin against heat denaturation, despite the fact that they are apparently properly folded.

DISCUSSION

α-Crystallins and small HSPs are members of the same protein superfamily. All members of the family are characterized by the presence of a common protein domain of about 90 residues, followed by an extension of variable length [4,6]. In Wistow's model the common domain is preceded by a more variable N-terminal domain. The homologous C-terminal domain is composed of two similar structural motifs (Fig. 1) [20]. Most probably,

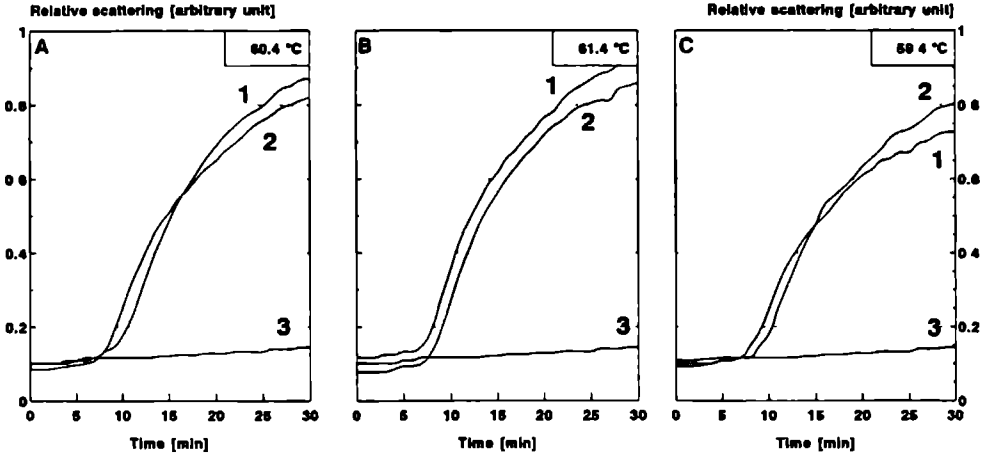


Figure 8: Heat-induced aggregation of βL-crystallin in the absence and presence of αA2Dt, αB2Dt and HSP2Dt. In each experiment 0.16 mg of βL-crystallin in a final volume of 0.4 ml was used. Aggregation of βL-crystallin was measured either alone (curve 1 in panel A, B and C), in the presence of 0.05 mg of recombinant αA-crystallin (panel A, B and C, curve 3) or in the presence of 0.092 mg of αA2Dt (panel A, curve 2), 0.11 mg of αB2Dt (panel B, curve 2) and 0.074 mg of HSP2Dt (panel C, curve 2). The incubation temperature is indicated at the top of each panel.

the conserved C-terminal domain and extension are responsible for the common structural and functional properties of the α-crystallin/small HSP family. In this work we addressed the question whether the secondary and quaternary structure of this C-terminal sequence is conserved. Therefore, the C-terminal domain plus tail (2Dt) of rat αA-crystallin, calf αB-crystallin and mouse HSP25 were isolated and subjected to gel permeation chromatography, crosslinking and CD spectroscopy after de- and renaturation.

Far UV CD spectroscopy indicated that the three C-terminal domains and tails are structurally well organized, the secondary structure elements being predominantly β-sheet. At most 10% of α-helix is present. The same distribution of secondary structure elements is found in the proteins from which the C-terminal domains and tails are derived. This is in agreement with the proposal that the α-crystallin/small HSP subunits are composed of two domains with similar structural motifs [20]. The CD spectra thus indicate that the C-terminal fragments are properly folded and can exist as independent structural units. The fact that the CD spectra of all protein fragments resemble each other, suggests that not only the primary structure but also the secondary structure has evolutionarily been conserved, as could be expected.

The conservation of the interactions between these protein fragments, however, is not obvious. Earlier, we showed that αA2Dt forms dimers or tetramers [21]. Gel permeation analysis of αB2Dt and HSP2Dt now revealed that these two domains form larger aggregates. αB2Dt is most probably a heterogeneous population of differently sized aggregates, the largest aggregate consisting of about 18-20 subunits. HSP2Dt forms smaller but more homogeneous aggregates, comprising approximately 12-13 subunits. Covalent crosslinking experiments, however, showed that HSP2Dt has a strong preference for dimers or tetramers and almost no higher crosslinking products are formed. On the other hand, αB2Dt is easily crosslinked, resulting in the formation of high molecular weight crosslinking products. This apparent discrepancy could be due to the absence of free ε-amino groups on certain interacting surfaces, which would result in poor crosslinking. It is noteworthy that HSP2Dt, forming small oligomers of two or four subunits upon crosslinking, contains only 6 and 7 lysines, respectively, whereas αB2Dt contains 10 lysine residues. Lysines are present at positions 6 and 16 in αA2Dt and αB2Dt, but not in HSP2Dt. It might be that amino acids in the region 1-20 are involved in interactions between tetramers when larger aggregates are formed. This would explain why the subunits in HSP2Dt aggregates cannot be crosslinked by dimethyl suberimidate to polymers larger than tetramers. The fact that lysines are present at positions 29, 40, 58, 87/88 and at the very C-terminal end in both HSP2Dt and αB2Dt, indicates that the regions around these lysines are not involved in contacts between tetramers. By examining the positions of other lysine residues, it appears that the lysines corresponding to the positions 29, 40 and 86-88 are present in all three proteins. One or more of these lysine residues might thus be involved in crosslinking within tetramers. It is conceivable that the region corresponding with positions 25-50 plays a role in the interactions between subunits within a tetramer.

Wherever the positions of the interactive surfaces may be, the present results can be taken to fit the rhombic dodecahedric model for α-crystallin [22]. In this model it is proposed that α-crystallin and small HSP aggregates are made up of tetrameric building blocks. The interactions between subunits within a tetramer differ from those between tetramers. The fact that HSP2Dt can be crosslinked to tetramers, while forming *per se* large aggregates is

indeed an indication that two types of contacts exist. In this model it is also proposed that α-crystallin and small HSP aggregates may have no single quaternary structure, but at least one stable structure, the rhombic dodecahedron. The fact that αB2Dt apparently is a heterogeneous population of aggregates (Fig. 6) may in fact reflect that α-crystallin and small HSP aggregates are dynamic structures, with several possibilities for oligomerization.

Recently, it has been discovered that both α-crystallin and HSP25 have *in vitro* chaperone activity [13-15]. From the sequence comparison of α-crystallins and small HSPs it is clear that the C-terminal domain is the essential conserved feature of this family [6]. It thus is likely that the chaperone properties of these proteins are primarily associated with this conserved region, and modulated by the variable N-terminal domain. Also the fact that the C-terminal domain with tail can form independent orderly structures, and that this region is probably located at the exterior of the native aggregates [22], made it relevant to test whether these structures exhibit chaperone activity. Moreover, also in the case of HSP70, the protein-binding activity is associated with a separate domain of the protein [36]. It turned out that none of the three polypeptides revealed protection activity. Apparently, other or additional parts of the protein are required for heat protection activity. It is yet unclear which molecules are chaperoned by α-crystallin and the small HSPs *in vivo* and which protein regions are involved in interactions between these molecular chaperones and their substrates. A more detailed study in which the α-crystallin subunits and HSP25 are less drastically changed, may give information about the elements necessary for substrate binding and chaperone activity.

REFERENCES

1. Bloemendal, H. (1981) Molecular and cellular biology of the eye lens, John Wiley and Sons, New York
2. Harding, J. J. and Crabbe, M. J. C. (1984) The Eye, Academic Press, New York
3. Wistow, G. J. and Piatigorsky, J. (1988) Annu. Rev. Biochem. 52, 479-504.
4. Ingolia, T. D. and Craig, E. A. (1982) Proc. Natl. Acad. Sci. USA 79, 2360-2364.
5. Lindquist, S. and Craig, E. A. (1988) Ann. Rev. Genet. 22, 631-677.
6. De Jong, W. W., Leunissen, J. A. M., and Voorter, C. E. M. (1993) Mol. Biol. Evol. 10, 103-116.
7. van den Heuvel, R., Hendriks, W., Quax, W., and Bloemendal, H. (1985) J. Mol. Biol. 185, 273-284.
8. Kato, K., Shinohara, H., Kurobe, N., Goto, S., Inaguma, Y., and Ohshima, K. (1991) Biochim. Biophys. Acta 1080, 173-180.
9. Kato, K., Shinohara, H., Kurobe, N., Inaguma, Y., Shimizu, K., and Ohshima, K. (1991) Biochim. Biophys. Acta 1074, 201-208.
10. Bhat, S. P. and Nagineni, C. N. (1989) Biochem. Biophys. Res. Comm. 158, 319-325.
11. Dubin, R. A., Wawrousek, E. F., and Piatigorsky, J. (1989) Mol. Cell. Biol. 9, 1083-1091.
12. Klemenz, R., Fröhli, E., Steiger, R. H., Schäfer, R., and Aoyama, A. (1991) Proc. Natl. Acad. Sci. USA 88, 3652-3656.
13. Horwitz, J. (1992) Proc. Natl. Acad. Sci. USA 89, 10449-10453.
14. Merck, K. B., Groenen, P. J. T. A., Voorter, C. E. M., De Haard-Hoekman, W. A., Horwitz, J., Bloemendal, H., and De Jong, W. W. (1993) J. Biol. Chem. 268, 1046-1052.
15. Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) J. Biol. Chem. (in press)
16. Bindels, J. G., Siezen, R J., and Hoenders, H. J. (1979) Ophthalmic Res. 11, 441-452.
17. Tardieu, A., Laporte, D., Licinio, P., Krop, B., and Delaye, M. (1986) J. Mol. Biol. 192, 711-724.
18. Augusteyn, R. C. and Koretz, J. F. (1987) FEBS Lett. 222, 1-5.
19. Walsh, M. T., Sen, A. C., and Chakrabarti, B. (1991) J. Biol. Chem. 266, 20079-20084.

20. Wistow, G. (1985) FEBS Lett. 181, 1-6.
21. Merck, K. B., De Haard-Hoekman, W. A., Oude Essink, B. B., Bloemendal, H., and De Jong, W. W. (1992) Biochim. Biophys. Acta 1130, 267-276.
22. Wistow, G. J. (1993) Exp. Eye Res. (in press)
23. Boyer, H. W. and Poulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459-472.
24. Gibson, T. J. (1984) Studies on the Epstein-Barr virus genome. Ph.D. Thesis, Cambridge University,
25. Gaestel, M., Gross, B., Benndorf, R., Strauss, M., Schunk, W., Kraft, R., Otto, A., Bohm, H., Stahl, J., Drabsch, H., and Bielka, H. (1989) Eur. J. Biochem. 179, 209-213.
26. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1989) Methods Enzymol. 185, 60-89.
27. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
28. De Jong, W. W., Zweers, A., Versteeg, M., and Nuy-Terwindt, E. C. (1984) Eur. J. Biochem. 141, 131-140.
29. Gaestel, M., Schröder, W., Benndorf, R., Lippmann, C., Buchner, K., Hucho, F., Erdmann, V. A., and Bielka, H. (1991) J. Biol. Chem. 266, 14721-14724.
30. Cohen, L. H., Westerhuis, L. W., De Jong, W. W., and Bloemendal, H. (1978) Eur. J. Biochem. 89, 259-266.
31. Greenfield, N. and Fasman, G. D. (1969) Biochem. 8, 4108-4116.
32. Schägger, H. and von Gebhard, J. (1987) Anal. Biochem. 166, 368-379.
33. Lavoie, J., Chrétien, P., and Landry, J. (1990) Nucleic Acids Res. 18, 1637.
34. Hickey, E., Brandon, S. E., Potter, R., Stein, G., Stein, J., and Weber, L. A. (1986) Nucleic Acids Res. 14, 4127-4145.
35. Carper, S. W., Rocheleau, T. A., and Storm, F. K. (1990) Nucleic Acids Res. 18, 6457.
36. Gething, M. -J. and Sambrook, J. (1992) Nature 355, 33-45.
37. Carver, J. A., Aquilina, J. A., Truscott, R. J. W., and Ralston, G. B. (1992) FEBS Lett. 311, 143-149.

CHAPTER 5

Exploring the aggregation and chaperone behaviour of αA-crystallin by site-directed mutagenesis

Exploring the aggregation and chaperone behaviour of αA-crystallin by site-directed mutagenesis

To explore the structure and chaperone activity of α-crystallin, five point-mutants of bovine αA-crystallin were generated by oligonucleotide-directed mutagenesis. The mutant αA(D69S), in which an evolutionarily well-conserved charged residue is replaced, differed considerably from the wild-type with regard to secondary structure. Its heat protection capacity was substantially decreased as compared with wild-type αA-crystallin. It appears that Asp-69 is important for stabilization of the tertiary structure, by ionic interaction, and that disruption of the structure leads to loss of heat protection activity. Four other mutations were aimed at disturbing hydrophobic intersubunit interactions. Hydrophobic residues Leu-37, Phe-39, Val-72 and Phe-74 are predicted to be involved in contacts between subunits. They were replaced by uncharged hydrophilic residues. The four mutants form smaller aggregates than the wild-type, although their secondary structures are still intact. These mutations did not seem to significantly affect the heat protection activity. The four hydrophobic residues thus might indeed play a role in the interaction between subunits.

INTRODUCTION

α-Crystallin is a major protein component of the vertebrate eye lens. It is a large aggregate of about 800 kDa and it is composed of two types of subunits, αA- and αB-crystallin, that occur in low amounts in many extralenticular tissues [1-4]. Both subunits have a molecular mass of about 20 kDa and are homologous with each other and with the small heat shock proteins (HSPs)[1] [5-7]. Although hardly any data concerning the three-dimensional structure of α-crystallin and the small HSPs is available, gradually some aspects of their functions are being revealed. αB-Crystallin is, like the small HSPs, heat-inducible and relocates from the cytoplasm to the nucleus under heat shock conditions [8]. Both bovine α-crystallin and mouse HSP25 act as molecular chaperones [9-11]. The structures of α-crystallin and the small HSPs seem to be related as well [9]. α-Crystallin as well as the small HSPs exist as large aggregates [12-15], and evidence has been provided that the arrangements of subunits resemble each other in both types of aggregates and that the secondary structures are very similar [9]. The common functional features of α-crystallin and the small HSPs obviously relate to the structural characteristics shared by these proteins. Therefore, it is of importance to solve the three-dimensional structure of the members of this protein family. However, until now, only tertiary structure predictions and quaternary structure models are available. Wistow [16] proposed the overall structure of α-crystallin to consist of a globular N-terminal domain of two symmetry-related motifs and a somewhat larger C-terminal domain, also of two motifs, with an exposed C-terminal tail. The four motifs are characterized by 3 residues (Asp/Glu, Pro and Gly) that are well conserved, especially in the C-terminal domains of the α-crystallin/small HSP protein family (Fig. 1). These residues might be of structural importance. One of these is Asp-69 in bovine αA-crystallin, which is surrounded by several other charged amino acid resi-

[1]The abbreviations used are: HSP, heat shock protein; PMSF, phenylmethylsulfonyl chloride; IPTG, isopropylthio-β-D-galactoside.

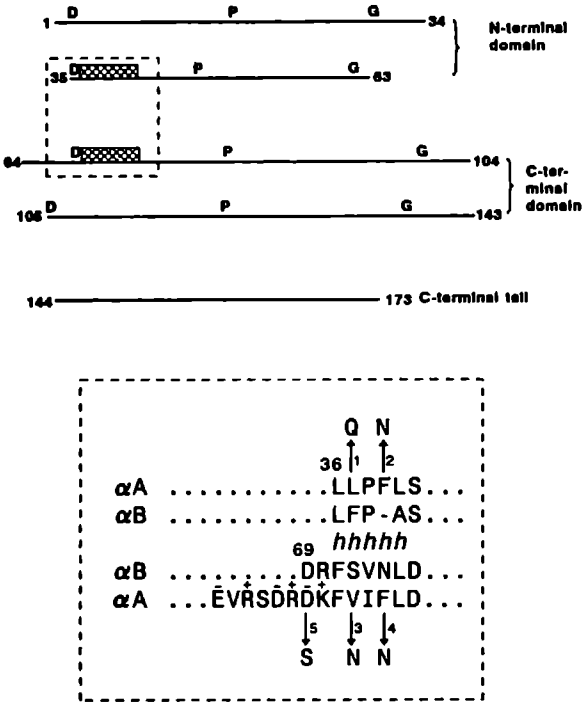


Figure 1: Localization of the mutations in the domain structure of αA-crystallin. The structure of αA-crystallin is displayed in five parts, the first two corresponding with the two putative motifs in the N-terminal domain, the next two with the putative motifs in the C-terminal domain, and the last one representing the C-terminal extension, as proposed by Wistow [16]. The conserved aspartyl, prolyl and glycyl residues of potential structural importance in the four motifs are indicated. The two stretches of five hydrophobic residues are represented by checked boxes. In the magnified panel, these regions of αA-crystallin and the corresponding residues of αB-crystallin are aligned to show the introduced mutations. The residues that are part of the five residue hydrophobic stretches in αA-crystallin are indicated with an *'h'*. The arrows denote the mutations and the corresponding oligonucleotide numbers.

dues (Fig. 1). It can be envisaged that salt bridges between these residues are important for the stabilization of the tertiary structure.

Several quaternary structure models for α-crystallin have been proposed, including a three-layer arrangement [17,18], a micelle-like conformation [19], a combination of these two [20] and lately a rhombo-dodecahedric arrangement of subunits [21]. The nature of interaction between the subunits and the residues involved, are still unknown.

To address the question whether Asp-69 is important for the structure and proper functioning of αA-crystallin and to find residues involved in intersubunit interactions, five site-directed mutants of αA-crystallin were made. Asp-69 was replaced by the uncharged amino acid serine. In the other four cases, the mutation concerned a single hydrophobic residue that was replaced by a hydrophilic, uncharged residue. By means of gel permeation chromatography, the effect on aggregate size was determined. Far UV CD spectroscopy was

used to analyse the correct folding into ordered secondary structure. The functional integrity of the mutants was assessed on basis of this ability to protect βL-crystallin against heat denaturation. The results demonstrate that even in the absence of tertiary structure data, relevant information about the structural and functional roles of particular residues can be obtained.

MATERIALS AND METHODS

Bacterial strains, plasmids and reagents

Escherichia coli strains HB101 [22] and JM105 [23] were used as recipients in the cloning experiments. *E.coli* TG1 [24] was used for preparation of single-stranded template DNA. For overproduction of wild-type proteins *E.coli* strain B BL21(DE3) was used [25]. Plasmids used in the cloning experiments were pET8c [25] and pGEMZf5(+) (Promega). Restriction enzymes, calf intestine alkaline phosphatase, T4 DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories (BRL). IPTG, ampicillin, chicken egg white lysozyme, DNAase and proteinase inhibitors were obtained from Sigma. Oligonucleotides were synthesized using a Cyclone Plus DNA synthesizer from Milligen/Biosearch.

Construction of expression plasmids of mutant αA-crystallins

The expression clone pET8cαA [26], which contains the cDNA encoding bovine αA-crystallin, was used as the starting material for the construction of five site-directed mutants. A 325 base pair fragment, coding for residues 1-108 of αA-crystallin was obtained by NcoI-digestion of pET8cαA and inserted into the NcoI-site of pGEM5Zf(+) in such an orientation that the coding strand was replicated. The 325 base pair fragment was mutated according to Taylor *et al.* [27], using the oligonucleotide-directed *in vitro* mutagenesis system of Amersham. The five mutants were made, using the following non-coding oligonucleotides: AGGAAGGGCTGCAGTCG (1), GTGGAGGACAGGTTGGGCAGCAGGT (2), ATCCAGGAAGATGTTAAACTTGTCCCGG (3), CTTCACATCCAGGTTGATGACAAACTTG (4) and AAGATGACAAACTTAGATCTGTCGGATCGGA (5), resulting in the mutations L37Q, F39N, V72N, F74N and D69S, respectively. To obtain the mutant αA(V72N) the procedure

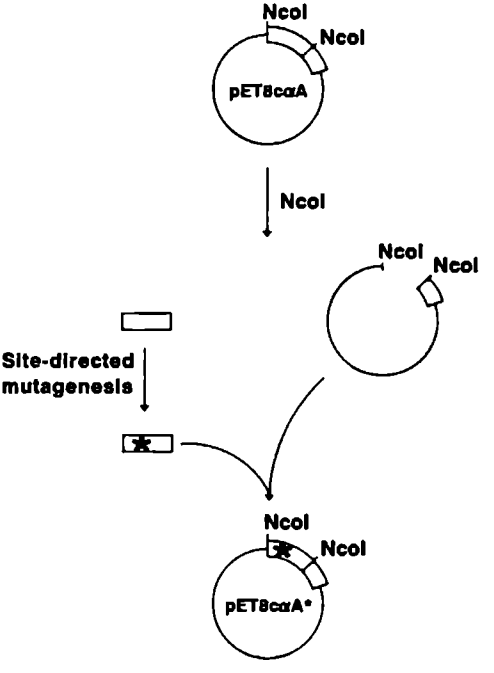


Figure 2: Strategy for the site-directed mutagenesis of αA-crystallin. A 325 base pair fragment was removed from the expression construct pET8cαA by NcoI-digestion. The 325 base pair fragment was mutated at the desired position according to Taylor *et al.* [27], using the five oligonucleotides as described in Materials and Methods. The mutated fragment was then cloned back in the NcoI-digested pET8cαA vector, resulting in the construct pET8cαA*. The asterisk indicates the presence of a mutation.

of the *in vitro* mutagenesis kit of Amersham had to be modified. Since primer 3 contains an NciI-site, we used PvuI in the nicking reaction, instead of NciI. The use of PvuI had no influence on the efficiency of the mutagenesis reaction. In all cases the efficiency was more than 90%. The mutated 325 base pair fragments were sequenced by the dideoxynucleotide method [28] to confirm the presence of the desired mutation and the absence of unwanted changes. The mutated 325 base pair fragments were then cloned back in the truncated expression plasmid.

Expression and purification of wild-type and mutant αA-crystallins

The wild-type and mutated expression constructs were transformed in the host *E.coli* B BL21(DE3). Expression of wild-type and mutant αA-crystallins, and preparation of the water-soluble fraction were performed essentially as described earlier [26]. Only the amount of lysozyme, used for the lysis of *E.coli* was reduced tenfold to 0.1 mg/ml, to limit the contamination of the *E.coli* lysate with lysozyme. The efficiency of the lysis was not affected by the reduction of the lysozyme concentration. All buffers used, as well as the water for dialysis, contained the following additives: 20 μM PMSF, 100 μM EDTA, 0.02 % β-mercaptoethanol (v/v), 40 μg/l bacitracin and 30 μg/l benzamidin. Lyophilized protein (30-100 mg) was dissolved in starting buffer (50 mM sodium phosphate, pH 6.8) and loaded on a Fast Flow DEAE-Sepharose column (Pharmacia-LKB), equilibrated with starting buffer. A linear gradient from 50 mM to 400 mM sodium phosphate at pH 6.8 (2.6 mM phosphate/min) was used to elute the proteins at a linear flow rate of 1.5 cm/min.

Reaggregation

Before gel permeation analysis, approximately 0.5 mg of wild-type or the mutant αA-crystallins were denatured in 4 ml of 6 M urea for 2 h at 4°C. The denatured samples were then dialysed against the appropiate gel permeation elution buffer. For far UV CD spectroscopy and heat protection experiments, protein samples were refolded as follows: lyophilized samples were dissolved in 6 M urea and diluted with the appropriate buffer to a concentration of 1 M urea and 1 mg/ml of protein, dialysed against water and lyophilized. All solutions used for reaggregation contained 0.02 % of β-mercaptoethanol (v/v) and proteinase inhibitors as indicated above.

Gel permeation analysis

Two different gel permeation columns were used. The Superose 6 HR 10/30 prepacked column (Pharmacia-LKB) was equilibrated with a buffer containing 50 mM sodium phosphate (pH 7.5), 50 mM NaCl and 1 mM EDTA, and elution was performed at 0.50 ml/min. The TSK G4000 SWXL-column (300x7.8 mm) (TosoHaas), to which a guard column (40x7.8 mm) was coupled, was equilibrated with a 150 mequiv. phosphate buffer (pH 6.8) [18] and eluted with a flow of 0.80 ml/min. In both cases, samples of approximately 25 μg were applied and absorbance was monitored at 225 nm. The gel permeation columns were calibrated with the high molecular mass calibration kit of Pharmacia-LKB.

Far UV circular dichroism measurements

These were performed using a Jasco Model 600 spectropolarimeter. A 0.25 mm pathlength cell was used. Reaggregated protein samples were measured in a 50 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl [9]. Each spectrum represents an average of 4 to 16 repetitive scans. The α-helix content was estimated according to Greenfield and Fasman [29].

Heat protection assay

The capacity of wild-type and mutant αA-crystallins to protect ßL-crystallin against heat-induced aggregation was assayed as described before [10]. In all experiments 0.16 mg of βL-crystallin in the presence or absence of wild-type and mutant αA-crystallin was dissolved in the same buffer as used for the CD measurements and centrifuged at 20,000 rpm for 20 min. The final volume in each experiment was 0.4 ml. Protein concentrations were determined on basis of amino acid analysis. Protection against heat denaturation of 0.25 mg of horse liver alcohol dehydrogenase (Boehringer) by wild-type and mutant αA-crystallins was performed at 44.3°C by measuring the increase in absorption at 360 nm in triplicate. One-hundred percent inhibition was defined as complete protection (no change in absorbance) during an incubation of 45 min.

RESULTS

Mutagenesis of bovine αA-crystallin

One of the goals of this study was to test whether the negatively charged conserved Asp-69 is important for the structure and function of bovine αA-crystallin. We therefore replaced Asp-69 by an uncharged polar residue of about the same size, namely serine. Additionally, we wanted to localize residues in the bovine αA-crystallin chain that play a role in the intersubunit interactions in the α-crystallin aggregates. Hydrophobic interaction sites are located at the surface of the subunits. Because hydrophobic residues involved in stabilizing tertiary core interactions within a subunit must be strongly conserved, we looked for those hydrophobic residues in αA-crystallin that are not strictly conserved. There are two stretches of five hydrophobic residues in bovine αA-crystallin, residues 36-40 and 71-75. In the latter the second and the fourth residues are not conserved as hydrophobic in αB-crystallin (Fig. 1) and also the sequence 36-40 is not well conserved. These regions might be β-strands that contribute to a domain core on one side and a domain or subunit surface at the other, like the a strand in motifs 2 and 4 in the β/γ-crystallins [30]. We replaced the second and fourth residue of the hydrophobic stretches by hydrophilic, non-charged residues (Fig. 1). Mutations were performed as described in Materials and Methods and are outlined in Fig. 2. The mutated 325 bp fragments were sequenced. Those clones that contained the desired mutation and did not have unwanted changes, were used for expression.

Expression and purification of wild-type and mutant αA-crystallins

Expression in *E.coli* and preparation of the water-soluble fraction of bacterial lysates was done as described earlier [26]. Expression levels of wild-type and mutant αA-crystallins were between 20 and 50%,

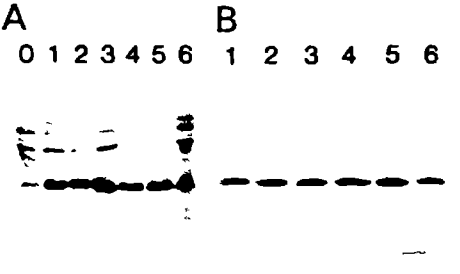


Figure 3: Expression in *E.coli* and purification of wild-type and mutant αA-crystallins. Sodium dodecylsulfate polyacrylamide gel electrophoresis patterns of (A) the water-soluble fraction of *E.coli* BL21(DE3) overexpressing wild-type αA-crystallin before (lane 0) and after induction by IPTG (lane 1), αA-crystallin mutants 1-5 after induction by IPTG (lanes 2-6, respectively) and of (B) wild-type αA-crystallin and its mutants 1-5 (lanes 1-6, respectively) after purification on a Fast Flow DEAE-Sepharose column.

as estimated by gel scanning (Fig. 3A). In all cases the mutants were predominantly in the water-soluble fraction of the lysates. The purification of wild-type and mutant αA-crystallins from the water-soluble fraction was accomplished by ion-exchange chromatography on a Fast Flow DEAE Sepharose column in a gradient of sodium phosphate. A typical elution pattern is shown in Fig. 4. The fractions were pooled as indicated in Fig. 4. In this way, 90-98% pure wild-type and mutant αA-crystallins could be obtained (Fig. 3B).

Aggregate size of the mutants

The aggregate size of wild-type and mutant αA-crystallins was estimated by means of gel permeation chromatography on calibrated columns. Prior to chromatography, the purified samples were denatured in 6M urea and renatured by dialysis against the appropriate gel permeation buffer. Without this reassociation step the elution volumes were not reproducible for different preparations of one mutant. By refolding all αA-crystallin variants under the same conditions, highly reproducible results could be obtained. Two different preparations of all mutants and the wild-type were analysed in duplo or triplo by gel permeation chromatography on two different columns. The elution patterns of the αA-crystallin variants on the TSK column are shown in Fig. 5. All mutants, except αA(D69S) have larger elution volumes than the wild-type. The elution volumes could be measured with errors of

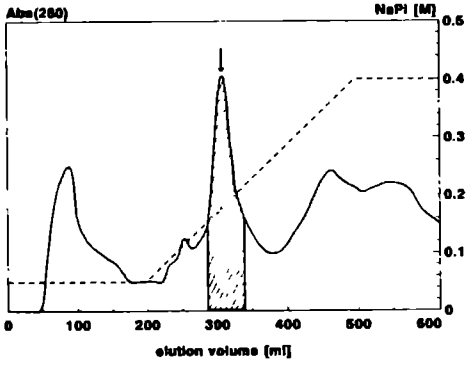


Figure 4: Elution pattern of αA(F39N) on Fast Flow DEAE-Sepharose. Solid line: absorbance profile (280 nm, arbitrary units) of the water-soluble fraction of *E.coli* αA(F39N). Broken line: phosphate gradient [M] with which the proteins were eluted. The arrow indicates the position of the mutant. The pooled fractions are indicated by the hatched area. Wild-type and the other mutant αA-crystallins yielded comparable elution patterns.

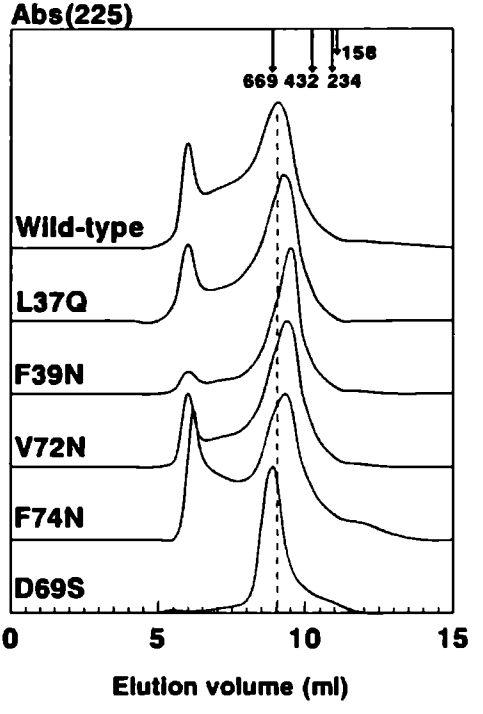


Figure 5: Gel permeation analysis of wild-type and mutant αA-crystallins. Absorbance profiles are presented after TSK G4000 SWXL chromatography of wild-type and mutant αA-crystallins. The first peak contains high molecular weight aggregates, the second peak represents the low molecular weight aggregates. The position of wild-type αA-crystallin is indicated by the broken line. Elution positions of gel permeation markers (in kDa) are indicated at the top of the figure.

less than 2%. In Table I the elution volumes and corresponding molecular masses are presented. Clearly, the calculated molecular mass of αA-crystallin aggregates is strongly dependent on the type of column on which the analysis is carried out. However, the molecular mass of the mutants relative to that of wild-type αA-crystallin is reproducible and not dependent on the gel permeation column used. The mutants in which hydrophobic residues were replaced (mutants 1-4) form considerably smaller aggregates, indicating that the mutated residues are involved in interactions between subunits. Mutant 5 (αA(D69S)) forms an aggregate that is slightly larger than that of the wild-type. The replacement of a conserved charged residue by an uncharged amino acid (D69S) apparently affects aggregate formation to a much lesser extent. However, the secondary structure of mutant 5 might be affected. To check whether or not the secondary structure of the mutants is still intact, we performed far UV CD experiments. Additionally, a 'chaperone assay' was carried out to assess the maintenance of functional activity.

Far UV CD spectroscopy

In Fig. 6 the far UV CD spectra of all five mutants compared with wild-type αA-crystallin are shown. Only the spectra of αA(V72N) and αA(F74N) are identical to that of wild-type αA-crystallin (panels C and D, respectively). The far UV CD spectrum of αA(F39N) slightly differs from that of the wild-type, its minimum being shifted to a somewhat lower wavelength (panel B). The spectrum of αA(L37Q) is different from that of wild-type (panel A). Its ~218 nm minimum is shifted to a higher wavelength and some intensity is lost at ~208 nm, indicating that the α-helix content might even be lower than in the wild-type. However, the secondary structure in mutants 1-4 is mainly β-sheet, with a maximum of 10% of α-helix, which is normal for αA-crystallin. In contrast, the CD spectrum of αA(D69S) is dramatically different from that of wild-type αA-crystallin. The pronounced minimum at ~208 nm indicates that αA(D69S) contains a considerable amount of α-helix (up to ~30%). The mutation D69S clearly affects the secondary structure, indicating that this charged residue (Asp-69) is important for retaining the proper structure of αA-crystallin.

Table I: Elution volumes and molecular masses of wild-type and mutant αA-crystallin.

	Superose 6B		TSK G4000SWXL	
	V_e (ml)	Mol.mass (kDa)	V_e (ml)	Mol.mass (kDa)
wt.	12.45	580	9.04	690
L37Q	12.80	510 (-12%)†	9.25	600 (-13%)
F39N	13.21	430 (-26%)	9.52	510 (-26%)
V72N	12.97	470 (-19%)	9.37	560 (-19%)
F74N	13.02	470 (-19%)	9.30	580 (-16%)
D69S	12.29	620 (+7%)	8.89	750 (+8%)

† Between brackets the change of the aggregate size relative to that of wild-type αA-crystallin

Inhibition of thermal aggregation of βL-crystallin by wild-type and mutant αA-crystallins.

Recently, α-crystallin was shown to act as a molecular chaperone *in vitro*. βL-Crystallin and other proteins are effectively protected against heat-induced denaturation in the presence of α-crystallin or the αA- and αB-homopolymers [10]. To determine whether the αA-mutants are as efficient in protecting βL-crystallin as wild-type αA-crystallin, the following experiment was carried out. A solution containing 0.16 mg of βL-crystallin in 0.4 ml was heated in the absence or presence of wild-type or mutant αA-crystallins. To be able to detect differences in heat protection capacity, the assay was carried out with low amounts of αA-crystallin, so that βL-crystallin is not fully protected. A molar ratio of approximately 1:100 of αA-crystallin to βL-crystallin, based on molecular masses of 600 and 60 kDa, respectively, was used in the assay. However, it turned out that at low concentrations of αA-crystallin to βL, the system is extremely sensitive, and sometimes a change of 1°C or less, or very small differences in the protein concentration have dramatic effects on the kinetics of aggregation. Therefore, it is not possible to detect minor differences in heat protection activity between the mutants. In Fig. 7 the heat protection curves for equal weights of wild-type or mutant αA-crystallins (0.016 mg) are depicted. It is striking that αA(D69S) (Fig. 7E) is very inefficient in protecting βL-crystallin against heat, compared to the wild-type and other mutants. In Fig. 7F are the heat denaturation curves when larger amounts of αA(D69S) are added. Only at higher concentrations, this mutant exhibits some heat protection activity. A three to four times higher concentration of αA(D69S) is needed to achieve approximately the same heat protection activity as, for example, αA(V72N) (Fig. 7C). To provide additional evidence that αA(D69S) is less active indeed, the heat-protective capacity of wild-type αA-crystallin and several mutants, including αA(D69S) was compared in another experiment. Various amounts of wild-type and mutant αA-crystallins were tested for their ability to protect alcohol dehydrogenase against denaturation at 44.3°C (Materials and Methods). 0.5 μg of wild-type and mutant αA-crystallin were able to inhibit heat denaturation of alcohol dehydrogenase for 40%. 0.5 μg of αA(D69S), however, resulted in only 10% protection activity. 2 μg of αA(D69S) was needed to achieve 40% protection. Also αA(F39N) was

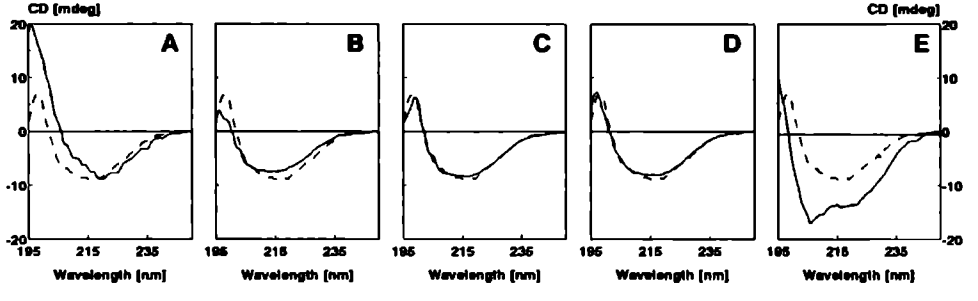


Figure 6: Far UV CD spectra of wild-type and mutant αA-crystallins. Panels A-E represent CD spectra of mutants 1-5 (solid lines), respectively, in comparison with the CD spectrum of wild-type αA-crystallin (broken lines). All CD spectra are normalized to a protein concentration of 0.5 mg/ml. The optical pathlength was 0.25 mm. The spectra are the average of 4 to 16 repetitive scans.

found to be less active with regard to heat protection (Fig. 7B). However 0.5 μg of this mutant resulted in 40% of heat protective capacity, like the wild-type and other mutants.

DISCUSSION

Despite the efforts that have been made to solve the structure of α-crystallin, little is known yet about its native conformation. Until now, it has not been possible to crystallize α-crystallin. Charge heterogeneity, brought about by the various post-translational modifications, might be one of the reasons for this problem [31]. Another possible cause is the dynamic and flexible nature of the α-crystallin aggregate [19,32]. To get information about the tertiary and quaternary structure of α-crystallin, indirect methods have been

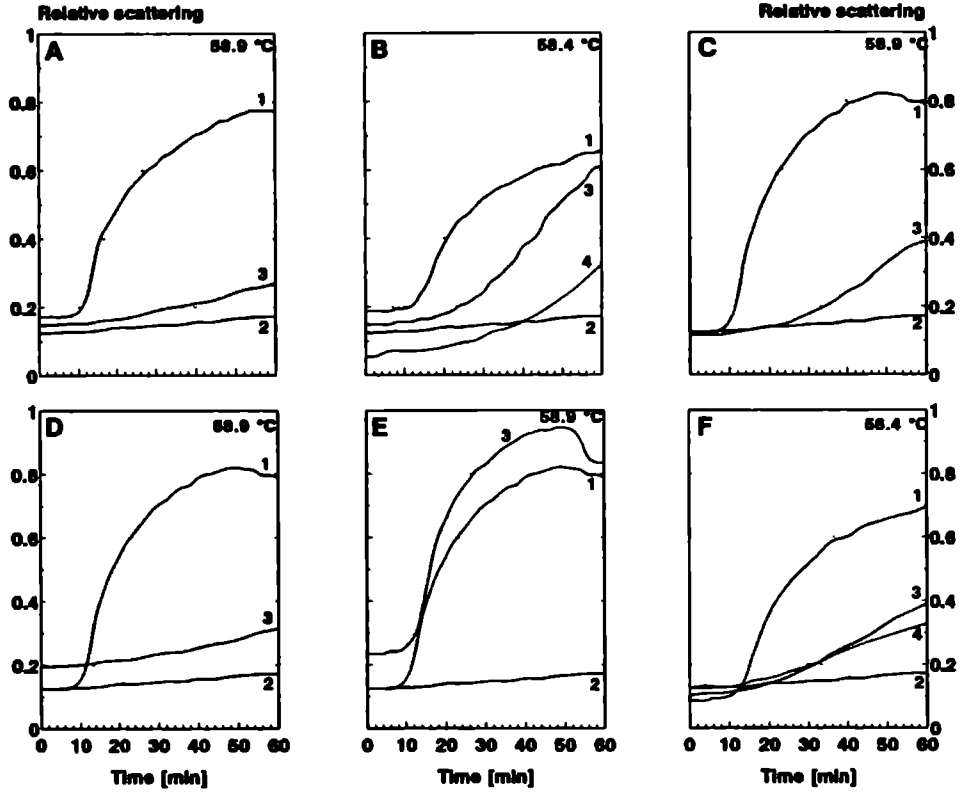


Figure 7: Heat-induced aggregation of βL-crystallin in the absence and presence of wild-type or mutant αA-crystallins. In each experiment 0.16 mg of βL-crystallin and 0.016 mg of wild-type or mutant αA-crystallins was used, unless indicated otherwise. The heating temperature was not identical for all mutants and is indicated in the Figure. Heat protection capacity of αA-crystallin mutants (mutant 1-5, curves 3 in panels A-E, respectively) is compared with that of wild-type αA-crystallin (curves 2). Curves 1 represent aggregation of βL-crystallin alone. Curve 4 in panel B represents the heat protection curve obtained when 0.02 mg of αA(F39N) is used. Panel F compares the heat protection capacity of increasing amounts of the mutant αA(D69S): 0.04 mg for curve 3 and 0.06 mg for curve 4.

employed, in which α-crystallin is modified and the effect on the structure measured. Many possibilities have been tested like sulphydryl and citraconic anhydride modification [33,34], and iodination of surface residues [35]. Site-directed mutagenesis is nowadays often used to obtain protein structure information, but had hitherto not been applied to α-crystallin. We now designed five site-directed mutants of bovine αA crystallin and measured the effect of the mutations on aggregate size, secondary structure and heat protection activity. Two kinds of mutants were made: one mutation concerned the replacement of a charged residue by an uncharged one, and another four mutants were replacements of hydrophobic by hydrophilic amino acids.

Electrostatic interactions are important for protein structure and function [e.g. 36-39]. The maintenance of a proper distribution of charged amino acid residues might therefore be an important factor in protein evolution. Leunissen *et al.* noticed that eye lens crystallins, and in particular α-crystallin, avoid changes in charge [40]. Indeed, charged interactions between the eye lens crystallins are supposed to be of extreme importance to maintain the transparency of the lens fiber cells. A close and even packing of these proteins is required to prevent light scattering [41,42]. By comparing the protein sequences of α-crystallins and small HSPs, it can be observed that certain charged residues are conserved throughout the protein family and in both protein domains (Fig. 1) [5]. One of these charged residues is aspartic acid at position 69 in bovine αA-crystallin, which is surrounded by pairs of oppositely charged residues (Fig. 1). Ion pairs at the surface of proteins are known to stabilize the tertiary structure and contribute to the thermostability of proteins [39,43,44]. It is therefore conceivable that Asp-69 is involved in stabilizing the tertiary structure. This aspartic acid residue was substituted by serine. UV CD spectroscopy established that αA(D69S) contains secondary structure elements, but the spectrum is quite different from that of wild-type αA-crystallin. The α-helix content is much higher in αA(D69S). Remarkably, despite the alteration in secondary structure, the quaternary structure does not seem to be affected. αA(D69S) still forms large aggregates, slightly larger than those of wild-type αA-crystallin. In fact, the viability of the structure is not grossly disrupted by this mutation, since αA(D69S) is still water-soluble.

The heat protection activity of αA(D69S) is considerably lower than that of the wild-type. Several possible reasons for the decreased protection capacity can be envisaged. The alteration of secondary structure elements, necessary for the binding to βL-crystallin (the stressed protein), might cause the observed decrease in heat protection activity. It might also be the negative charge itself that is important for the interaction of α-crystallin with βL-crystallin. Charged residues are mostly found on the surface of proteins and may be of importance for recognition and interactions between molecules [45,46], for instance the interaction of α-crystallin with cytoskeletal proteins [47-50]. Our results clearly demonstrate that Asp-69 is important for the structure and function of αA-crystallin.

The other four mutants replace hydrophobic residues by hydrophilic ones. The sites that form the protein interior are occupied mainly by nonpolar residues. The surfaces between secondary structure elements are almost entirely hydrophobic in character [51]. On the other hand, hydrophilic surfaces are almost always accessible to the solvent. Residues that are involved in aggregate formation, thus in intersubunit interactions, are not expected to form part of the hydrophobic core, but are present on the surface of a subunit. However, they should not be exposed to the solvent. Evolutionarily well conserved hydrophobic

residues are likely to form part of the hydrophobic core, whereas less conserved hydrophobic residues could be present at the subunit interface. Four hydrophobic residues of αA-crystallin, not conserved as hydrophobic in αB-crystallin and making part of a hydrophobic five-residue stretch, were chosen to be replaced by polar but uncharged residues. All four mutants differ from wild-type αA-crystallin by forming smaller aggregates, up to 26% smaller than the wild-type aggregate. These mutations had little or no effect on the secondary structure. In all four cases the secondary structure is primarily β-sheet, like in αA-crystallin. Also the chaperone activity is largely retained in the mutants 1-4. The conclusion we may draw from this study is that the replacements of L37, F39, V72 and F74 by glutamine or asparagine do not change the secondary structure and have no drastic effects on the chaperone activity. The aggregate size, however, is affected; all four mutants form considerably smaller aggregates than the wild-type. It thus may be that the above mentioned hydrophobic residues form part of interacting hydrophobic subunit surfaces.

An increased knowledge of the structure of members of the α-crystallin/small HSP family would contribute to a better understanding of their *in vivo* function. It is still unclear which molecules are chaperoned by α-crystallin and the small HSPs *in vivo*, under which circumstances and by what mechanisms. In contrast with members of the chaperonin 60 family, and the HSP70 and 90 families [52-55], α-crystallin and HSP25 do not seem to require ATP to refold γ-crystallin or protect βL-crystallin, citrate synthase and α-glucosidase [9-11]. It is therefore particularly interesting to unravel the structure-function relationship of the α-crystallin/small HSP family.

REFERENCES

1. Kato, K., Shinohara, H., Kurobe, N., Goto, S., Inaguma, Y. and Ohshima, K. (1991) Biochim. Biophys. Acta 1080, 173-180.
2. Kato, K., Shinohara, H., Kurobe, N., Inaguma, Y., Shimizu, K. and Ohshima, K. (1991) Biochim.Biophys. Acta 1074, 201-208.
3. Bhat, S.P. and Nagineni, C.N. (1989) Biochem. Biophys. Res. Comm. 158, 319-325.
4. Dubin, R.A., Wawrousek, E.F. and Piatigorsky, J. (1989) Mol. Cell. Biol. 9, 1083-1091.
5. De Jong, W.W., Leunissen, J.A.M., Leenen, P.J.M., Zweers, A. and Versteeg, M. (1988) J. Biol. Chem. 263, 5141-5149.
6. Ingolia, T.D. and Craig, E.A. (1982) Proc. Natl. Acad. Sci. USA 79, 2360-2364.
7. Lindquist, S. and Craig, E.A. (1988) Ann. Rev. Genet. 22, 631-677.
8. Klemenz, R., Fröhli, E., Steiger, R.H., Schäfer, R. and Aoyama, A. (1991) Proc. Natl. Acad. Sci. USA 88, 3652-3656.
9. Merck, K.B., Groenen, P.J.T.A., Voorter, C.E.M., De Haard-Hoekman, W.A., Horwitz, J., Bloemendal, H. and De Jong, W.W. (1993) J. Biol. Chem. 268, 1046-1052.
10. Horwitz, J. (1992) Proc. Natl. Acad. Sci. USA 89, 10449-10453.
11. Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) J. Biol. Chem. (In Press)
12. Collier, N.C., Heuser, J., Levy, M.A. and Schlesinger, M.J. (1988) J. Cell Biol. 106, 1131-1139.
13. Hockertz, M.K., Clark-Lewis, I. and Candido, E.P.M. (1991) FEBS Lett. 280, 375-378.
14. Arrigo, A.-P. and Welch, W.J. (1987) J. Biol. Chem. 262, 15359-15369.
15. Spector, A., Li, L.-K., Augusteyn, R.C., Schneider, A. and Freund, T. (1971) Biochem. J. 124, 337-343.
16. Wistow, G. (1985) FEBS Lett. 181, 1-6.
17. Bindels, J.G., Siezen, R.J. and Hoenders, H.J. (1979) Ophthalmic Res. 11, 441-452.
18. Tardieu, A., Laporte, D., Licinio, P., Krop, B. and Delaye, M. (1986) J. Mol. Biol. 192, 711-724.
19. Augusteyn, R.C. and Koretz, J.F. (1987) FEBS Lett. 222, 1-5.
20. Walsh, M.T., Sen, A.C. and Chakrabarti, B. (1991) J. Biol. Chem. 266, 20079-20084.
21. Wistow, G.J. (1993) Exp. Eye Res. (In Press)

22. Boyer, H.W. and Poulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459.
23. Yanish-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103-119.
24. Gibson, T.J. (1984) Studies on the Epstein-Barr virus genome. Ph.D. Thesis, Cambridge University, Cambridge.
25. Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1989) Methods Enzymol. 185, 60-89.
26. Merck, K.B., De Haard-Hoekman, W.A., Oude Essink, B.B., Bloemendal, H. and De Jong, W.W. (1992) Biochim. Biophys. Acta 1130, 267-276.
27. Taylor, J.W., Ott, J. and Eckstein, F. (1992) Nucleic Acids Res. 13, 8765-8785.
28. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
29. Dyson, H.J., Merutka, G., Waltho, J.P., Lerner, R.A. and Wright, P.E. (1992) J. Mol. Biol. 226, 795-817.
30. Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, I., Turnell, B. and Wistow, G. (1981) Nature 289, 771-777.
31. Siezen, R.J., Bindels, J.G. and Hoenders, H.J. (1978) Eur. J. Biochem. 91, 387-396.
32. van den Oetelaar, P.J., van Someren, P.F.H.M., Thomson, J.A., Siezen, R.J. and Hoenders, H.J. (1990) Biochemistry 29, 3488-3493.
33. Siezen, R.J., Coenders, F.G. and Hoenders, H.J. (1978) Biochim. Biophys. Acta 537, 456-465.
34. Bindels, J.G., Misdom, L.W. and Hoenders, H.J. (1985) Biochim. Biophys. Acta 828, 255-260.
35. Hendriks, W., Weetink, H., Voorter, C.E.M., Sanders, J., Bloemendal, H. and De Jong, W.W. (1990) Biochim. Biophys. Acta 1037, 58-65.
36. Matthews, J.B. (1985) Annu. Rev. Biophys. Biophys. Chem. 14, 387-417.
37. Akka, M. and Forsen, S. (1990) Proteins: Struct. Funct. Genet. 8, 23-29.
38. Perry, K.M., Onuffer, J J., Gittelman, M.S., Barmat, L. and Matthews, C.R. (1989) Biochemistry 28, 7961-7968.
39. Horovitz, A., Serrano, L., Avron, B., Bycroft, M. and Fersht, A.R. (1990) J. Mol. Biol. 216, 1031-1044.
40. Leunissen, J.A.M., van den Hooven, H.W. and De Jong, W.W. (1990) J. Mol. Evol. 31, 33-39.
41. Delaye, M. and Tardieu, A. (1983) Nature 302, 415-417.
42. Slingsby, C. (1985) Trends Biochem. Sci. 10, 281-284.
43. Perutz, M.F. (1987) Science 201, 1187-1191.
44. Barlow, D.J. and Thornton, J.M. (1983) J. Mol. Biol. 168, 867-885.
45. Rose, G.D., Geselowitz, A.R., Lesser, G.L., Lee, R.H. and Zehfus, M.H. (1985) Science 229, 834-838.
46. Miller, S., Janin, J., Lesk, A.M. and Chothia, C. (1987) J. Mol. Biol. 196, 641-656.
47. Longoni, S., Lattonen, S., Bullock, G. and Chiesi, M. (1990) Mol. Cell. Biol. 97, 121-128.
48. Chiesi, M., Longoni, S. and Limbruno, U. (1990) Mol. Cell. Biol. 97, 129-136.
49. Bloemendal, H., Berbers, G.A.M., De Jong, W.W., Ramaekers, F.C S., Vermorken, A.J.M., Dunia, I. and Benedetti, E.L. (1984) in: Interaction of crystallins with the cytoskeletal-plasma membrane complex of the bovine lens (Human cataract formation, Ciba Fnd. Symposium 106, pp. 177-186, Pitman, London.
50. Fitzgerald, P.G. and Graham, D. (1991) Curr. Eye Res. 10, 417-436.
51. Chothia, C. and Finkelstein, A. (1990) Annu. Rev. Biochem. 59, 1007-1039.
52. Ellis, R.J. and Van der Vies, S.M. (1991) Annu. Rev. Biochem. 60, 321-347.
53. Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33-45.
54. Hartl, F.U., Martin, J. and Neupert, W. (1992) Annu. Rev. Biophys. Biomol. Struct. 21, 293-322.
55. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K. and Hartl, F.U. (1992) Nature 356, 683-689.

CHAPTER 6

Characterization of anti-crystallin autoantibodies in patients with cataract

Characterization of anti-crystallin autoantibodies in patients with cataract

Anti-crystallin autoantibodies have often been demonstrated in the serum of healthy persons and, especially, patients with cataract. In no case, however, have the specific crystallin subunits been identified against which such antibodies are directed. This information would be of particular interest in view of the recent finding that several crystallin subunits occur constitutively outside the lens. To fill this gap, we analysed the sera of 15 patients with mature cataract by means of 1- and 2-dimensional immunoblotting. The circulating antibodies turned out to be directed against several ß- and γ-crystallin subunits. The types of subunits and the intensities of the responses varied considerably between patients. No or only occasional and very weak reactions were observed against the αA-, αB- and ßB2-crystallin subunits. These are in fact the only crystallins at present known to occur outside the lens in mammals. Our findings thus indicate that anti-crystallin autoantibodies are specifically directed against those crystallins that appear to be lens-restricted, while immunological tolerance would exist for the extra-lenticularly occurring crystallins.

INTRODUCTION

The eye lens crystallins have always been considered as organ-specific proteins *par excellence*. Studies of the causes and effects of autoantibodies against the crystallins were therefore undertaken and interpreted from this perspective. However, the notion of lens-specificity of the crystallins has drastically changed since the recent findings of extra-lenticular crystallins [e.g. 1-3]. There have been earlier indications that crystallins may occur in trace amounts in ocular tissues other than the lens [4]. This was often attributed to the fact that the lens capsule, especially in cataract, is not a perfect barrier for proteins [5, 6]. It now appears that in many species certain crystallins occur constitutively outside the lens [7,8]. In man and other mammals αB-crystallin is quite abundant in many tissues, notably in heart, striated muscle and kidney [1, 9]. Recently the presence of very low amounts of extra-ocular αA-crystallin has been demonstrated, most clearly in rat spleen and thymus [2]. Also βB2-crystallin has now definitely been shown to occur outside the lens in rat and cat (M. W. Head and R. M. Clayton, personal communication).

In the light of these findings the phenomenon of circulating autoantibodies against crystallins comes into a different context. The presence of anti-crystallin antibodies in up to about 50% of normal human sera has been reported in several studies [10-13], although not in others [14-17]. This no longer requires the assumption of leakage of crystallins from the lens to get in contact with the immune system. Increased levels of anti-lens antibodies in cataractous patients are usually reported [11-13, 16], although not always [17]. Again, the increased leakage of crystallins from cataractous lenses needs no longer to be associated with an autoimmune response against all crystallin subunits, because tolerance against the extra-lenticular crystallins may exist.

No studies have been published in which the specific crystallin subunits reacting with anti-lens autoantibodies have been characterized. Such a study can best be performed on sera of patients with mature cataract, where the immune system is likely to be exposed for long periods to crystallins leaking out of the lens. It should then reveal whether or not a pattern

can be discerned in which certain crystallin subunits are more autoimmunogenic than others. A more pronounced autoimmune response would be expected against crystallin subunits that do not normally occur outside the lens. To make an initial inventory of anti-crystallin autoantibodies we here report the characterization of 15 patient sera by one- and two-dimensional immunoblotting. Our results demonstrate that great individual variation exists in the autoimmune response against crystallins. Antibodies against most ß- and γ-crystallin subunits were frequently found, while no or hardly any antibodies against αA, αB- and ßB2-crystallin could be observed in this limited sample.

MATERIALS AND METHODS

Blood was obtained from 10 patients suffering from senile and presenile mature cataract, and from 5 patients with luxated lenses and secondary mature cataract. Blood from 3 persons without ophthalmological problems was used for controls. The blood was allowed to clot and the serum collected by centrifugation at 4°C. The sera were then stored at -20°C until use.

One-dimensional polyacrylamide gel electrophoresis (1D SDS-PAGE) of lens proteins was performed according to Laemmli [18]. Two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE) was performed as described by O'Farell [19]. The isofocusing gels contained ampholines (LKB, Uppsala, Sweden) in the pH ranges 6-8, 7-9 and 3½-10, mixed in a ratio of 3:3:10. The 1D gels, used for Western blotting, were loaded with total water-soluble lens proteins of calf or human fetal lens (25 μg per cm of gel), or with 0.5 μg per cm of gel of αA-, αB-, βB1- and βB2-crystallin each. The latter subunits were isolated from calf lenses according to de Jong *et al.* [20] and Berbers *et al.* [21]. 2D gels were loaded with 40 μg or 150 μg of the water-soluble fraction of either calf lenses (6 months) or fetal human lenses (130-200 days) for Western blotting and Coomassie Brilliant Blue (CBB) staining, respectively.

For Western blotting the proteins were transferred from the gel onto nitrocellulose, according to Towbin *et al.* [22]. Incubations of the nitrocellulose blots with the sera and the washing step just before the staining reaction were carried out overnight, to minimize aspecific reactions. Human sera were diluted hundred-fold before incubation. Peroxidase-conjugated goat anti-human immunoglobulin (Nordic, Tilburg, The Netherlands) was used as second antibody against the human immunoglobulins at a dilution of 1:1000 and in the presence of 0.5% BSA. The control antisera used were prepared in rabbits against bovine αA2 and αB2 (polyclonal, dilution 1:2000), bovine βB1 (polyclonal, dilution 1:500, [23]) and bovine βB2 (polyclonal, dilution 1:2000, provided by Dr. J. Horwitz, UCLA). As second antibodies against the control antibody, peroxidase-conjugated swine anti-rabbit immunoglobulin (Dakopatts, Denmark) was used at a dilution of 1:250 in the presence of 1% normal goat serum. Immunostained Western blots were post-stained with Ponceau S to identify the immunoreactive spots.

RESULTS

In order to get an initial idea of the titers and types of the anti-crystallin antibodies in the sera of cataract patients and in normal control sera, we performed Western blotting on total water-soluble calf and fetal human lens proteins, resolved by 1D SDS-PAGE. The sera of 15 patients and three controls were screened. The patient antisera consistently showed a stronger reaction than the controls, where only very

weak bands could be observed. Also among the patients the patterns and intensities of the reaction varied considerably (data not shown). These one-dimensional patterns do not distinguish between the specific crystallin subunits to which the antibodies in the sera are directed. To identify these subunits we had to resort to Western blots after 2D SDS-PAGE of calf lens proteins. The positions of the different calf lens crystallin polypeptides on 2D SDS-PAA gels are better known [24] than those of human. In order to identify the crystallin chains to which the antibodies are directed we selected 6 patient sera, which exhibited the strongest and most variable reaction on 1D immunoblots, to incubate 2D Western blots. The blots were stained until a clear pattern was obtained (Fig. 1, panels A2-7). The spots on the Western blots were identified by staining with Ponceau S, and by comparing the Ponceau S pattern with the corresponding CBB-stained gel (Fig. 1, panel A1). The results are summarized in Table I. The relative intensities of the spots of all identified crystallin chains are given, as well as those for three unknown protein components, indicated as 1, 2 and 3. Since the staining times were different for all blots, the intensities of spots on the different blots cannot be compared with each other, whereas spots on the same blot can. From the blots and from the table, one can see that there are great differences between the reaction patterns of the different sera. More or less strongly reacting in all six sera are ßA1, ßA4 and γs. Most others, like ßA3, γ and #2, are reacting in most but not all sera. Strikingly, none of the sera reacts with calf αA-, αB- and βB2-crystallin. This could in principle be due to differences between calf- and human crystallin sequences, although the polypeptides of human crystallins resemble very much the corresponding bovine ones. It would therefore be of interest to analyse the reaction of the antisera with the autologous, human crystallins, even though most of these are not identified on the 2D SDS-PAA gel pattern. However, the positions of human αA, αB and βB2 could easily be determined by immunostaining with specific antisera (Fig. 1, panel B). Blots, of the water-soluble fraction of fetal human lenses, resolved by 2D SDS-PAGE, were incubated with the six patient sera and stained until a clear pattern was obtained (Fig. 1, panels B2-7). To identify the immunoreacting crystallin chains, the blots were stained with Ponceau S. Only in one case (patient 9) a relatively weak reaction with αA could be observed. A very weak reaction with αB-crystallin was observed with the serum of patient 8. To ascertain the position of αB, the blots were post-stained with the polyclonal antibody

Table I:: Relative intensities of spots on calf lens immunoblots after treatment with human sera from patients with mature cataract.*

Fig. 1	2	3	4	5	6	7
Patient nr.	8	9	10	11	14	15
αA	-	-	-	-	-	-
αB	-	-	-	-	-	-
βA1	+	+	+	+	+	+
βA2	±	-	+	-	-	-
βA3	+	-	+	-	+	±
βA4	+	+	+	+	+	+
βB1	±	±	+	-	±	+
βB2	-	-	-	-	-	-
βB3	±	±	+	-	±	+
γ	+	+	+	-	+	+
γs†	+	+	+	±	+	+
1	-	+	+	-	±	+
2	-	+	+	-	+	+
3	-	+	+	-	+	+

* - no reaction, ± weak reaction, + clear reaction, ++ strong reaction.
† formerly βs

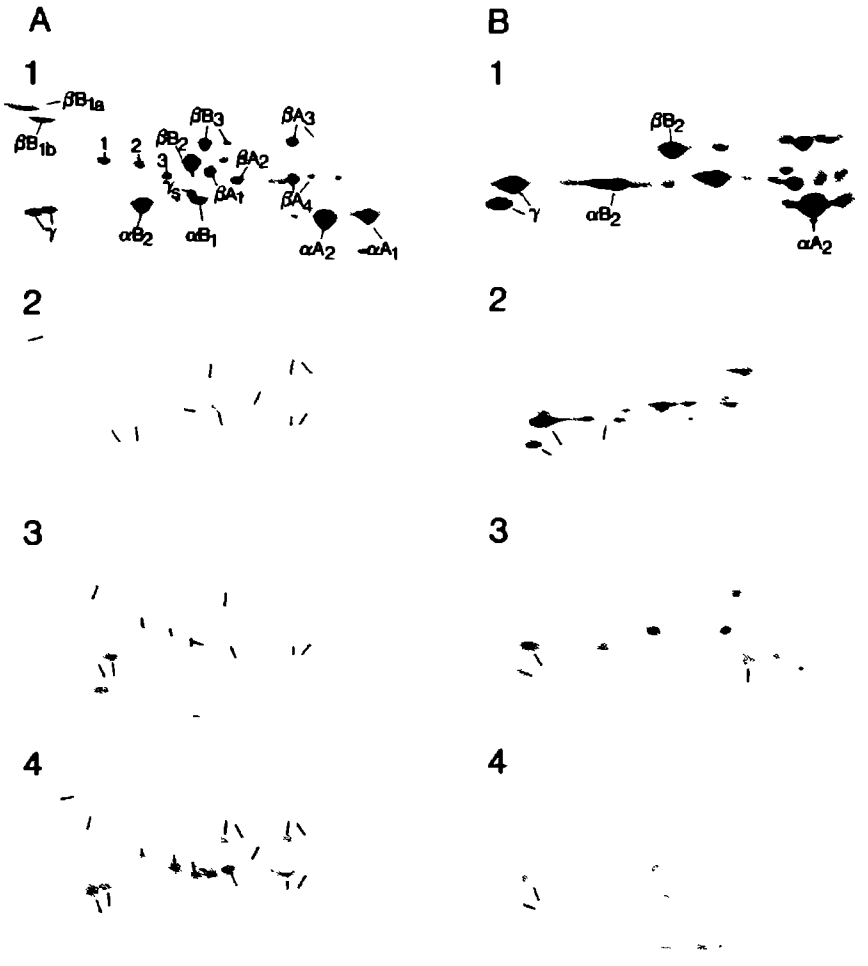


Fig. 1: Identification of crystallin subunits reacting with sera from cataract patients. 2D SDS-PAGE patterns (1) and their corresponding immunoblots (2-7), of the water-soluble proteins of calf lens (panel A) and of fetal human lens (panel B). The SDS-PAA gels (1) are stained with CBB. The corresponding immunoblots (2-7) were incubated with patient sera 8, 9, 10, 11, 14 and 15, respectively, and stained as described in Materials and Methods. On the CBB-stained patterns, the names of the bovine crystallin subunits are indicated [24]. The numbers 1, 2 and 3 indicate unidentified bovine crystallin subunits. ßB2, ßA3 and ßA4 have minor charge forms. The positions of especially ßB1 and γ may vary considerably on different blots. The relative position of γs on the blots is somewhat higher than on the CBB-stained gel. The human αA2, αB2 and ßB2 are identified by specific antisera, and the γ's are indicated on basis of their charge. The human ßB1 polypeptides are very basic and therefore not present on the 2D-gels. On the immunoblots, reacting subunits are indicated by lines, which are oriented in accordance with the pattern on the corresponding CBB-stained gel.

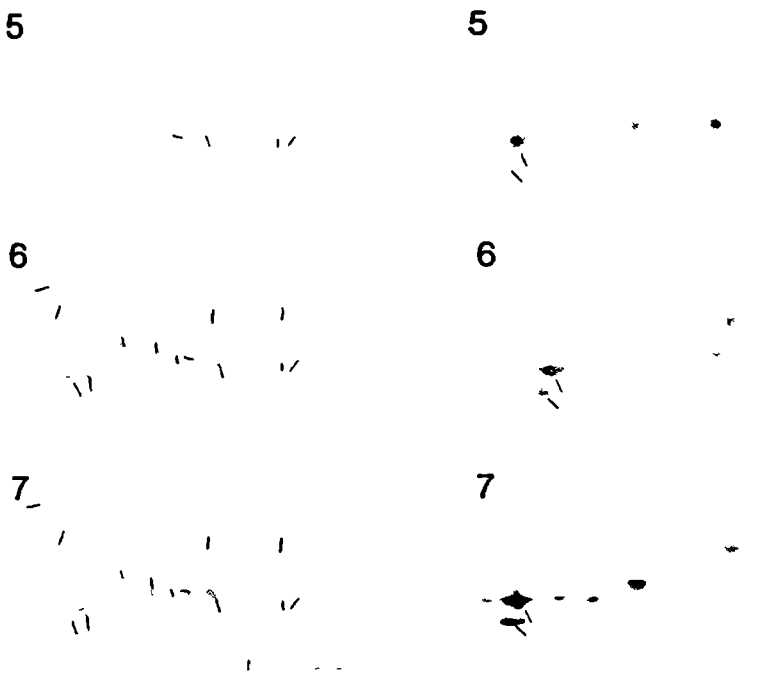


Fig. 1: Continued

spot appeared at a position where no prior staining with the human sera was observed. Finally, in no case a reaction with βB2 was found.

To confirm that autoantibodies against αA-, αB- and βB2-crystallin are absent or scarce in patient sera, we should have performed 2D blotting on the other 9 samples. Since this is quite laborious, and because not always enough serum was available, we obtained the desired information from 1D Western blots. These blots, containing a mixture of αA-, αB- and βB2-crystallin from calf lenses, were incubated with all 15 sera. As an internal positive control, βB1-crystallin, with which many of the sera react, was also present in the crystallin mixture. In Fig. 2 the results are presented. A clear reaction with the βB1-subunits is observed. In none of the cases a reaction, with αA occurs, except for patients 9 and 12, which exhibit a very weak response. Only in the case of patient 8, a very weak reaction with αB was visible on the blot. However, the reaction was so weak that it could not be reproduced on the photograph. In some cases (patients 5, 7 and 10) a very weak reaction with a protein at the position of βB2-crystallin is observed. This is not in agreement with the clear results obtained from the 2D Western blotting experiment, where never a reaction with βB2 was seen. It was found that a minor contamination was present in the βB2-crystallin fraction, as judged by denaturing iso-electrofocusing. The reacting antibodies most probably are directed against this contamination. The screening of these 15 sera thus seems to indicate that, in general, no antibodies against αA-, αB-, and βB2-crystallin are present in sera of cataract patients.

There are some remarkable features in our results. The intensities of the different

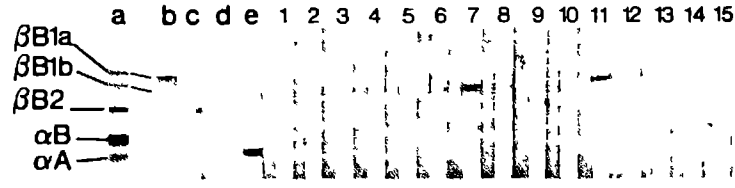


Fig 2: Reaction of specific crystallin subunits (αA, αB, ßB2 and ßB1) with sera of cataract patients. Western blot stripe, containing approximately equal amounts of bovine αA-, αB-, ßB2- and ßB1-crystallin, were incubated with the sera of all cataract patients. Lane a shows the CBB-stained pattern of the crystallin mixture, lanes b-e show Western blot strips incubated with antisera against ßB1, ßB2, αB and αA, respectively. Lanes 1-15 show the Western blot strips incubated with the sera of cataract patients with the corresponding number.

spots stained by a particular serum, while perfectly reproducible, do often not correlate well in the 2D patterns of calf and human lens proteins. For instance, a γ-crystallin spot is clearly present on the blot of human crystallins with serum 11, but not detectable on the calf lens blot (panels B5 vs. A5). Likewise, αA-crystallin reacts with serum 9 on the human blot, but is not detectable on the calf blot (panels B3 vs. A3). Remarkable, too, is the fact that on the calf blots the reaction with ßA1 is always more pronounced than the reaction with ßA3. This is unexpected since ßA3 is in fact the same polypeptide as ßA1, only having 15 N-terminal residues extra [25]. It can be envisaged that the antibodies present in the sera are primarily directed against an epitope containing the free N-terminus of ßA1-crystallin. This epitope would be altered by the N-terminal extension of ßA3-crystallin, making it less reactive.

DISCUSSION

In mature cataracts longstanding leakage of crystallins through the damaged capsule is likely to occur. The immune system is thus exposed to the crystallins which are normally sequestered in the lens. However, those crystallins that normally occur in small amounts outside the lens would be recognized as self, and therefore not normally evoke an autoimmune response. Only the truly lens-specific crystallins would be reacted against as non-self, thus triggering the formation of autoantibodies. Among our 15 sera of patients with mature cataracts there were no or only rare and weak reactions against αA-, αB- and ßB2-crystallins. It is striking that these proteins are precisely the only crystallins hitherto demonstrated to occur extra-lenticularly in mammals [1-3]. The absence of autoantibodies against these crystallins is in perfect agreement with the prediction that an autoimmune response would not normally be expected against crystallins that occur constitutively outside the lens.

In mammals no other crystallins than αA, αB and ßB2 have yet been observed outside the lens, although in chicken the ßB2 and ßA3/A1 crystallin mRNAs occur in the retina [3]. It is of course still possible that additional crystallins will turn out to be extra-lenticular in human. From an evolutionary point of view it is surprising that at least γs and one or more of the other γ-crystallins have not been found outside the lens. The divergence of the ß- and γ-crystallins probably preceeded the origin of the vertebrate eye [25]. Not only an ancestral ß-crystallin, but also the original γ-crystallins should therefore initially have had an extra-lenticular function, which might be expected to still be main-

tained in present-day vertebrates. However, considering the frequent and relatively pronounced autoimmune response against all γ-crystallins and almost all ß-crystalins in the patients tested, it appears that they are approached as if they are non-self. This indicates that these crystallins have not been in contact with the immune system at any time during development, and thus are most likely confined to the lens.

In several studies it has been reported that anti-crystallin antibodies are present in up to about 50% of normal human sera [10-13]. In sera of healthy control patients we could also detect autoantibodies, although the reactions were less intense as compared with patients suffering from cataract. We also observed an increase in intensity, with increasing age of the healthy controls. It should be noted that considerable variation exists in the types and intensities of autoantibodies in different patients. Some crystallins, evoke strong responses in some patients, but not in others (e.g. protein #2, ßA3). This is not too surprising in view of the poorly understood and seemingly capricious nature of the human autoimmune response in general. It should finally be mentioned that antibodies to α-crystallin have transiently been detected, by radioimmunoassay, in mice after lens rupture by needling [26]. Antibodies against α-crystallin, as well as ß- and γ-crystallins, were also reported, as determined by immunoblotting and enzyme-linked immunosorbent assay, in normal sera and at increased level in patients with senile cataract [27]. Whether these reports are really conflicting with our findings, or rather can be explained by differences in experimental approach, requires further study.

REFERENCES

1. Bhat S.P. and Nagineni C.N. (1989) Biochem. Biophys. Res. Commun. 158, 319-325.
2. Kato K., Shinohara H., Kurobe N., Goto S., Inaguma Y. and Oshima K. (1991) Biochim. Biophys. Acta 1080, 173-180.
3. Head M.W., Peter A. and Clayton R.M. (1991) Differentiation 48, 147-156.
4. Clayton R.M., Campbell J.C. and Truman D.E.S. (1968) Exp.Eye Res. 7, 11-29.
5. François J. and Rabaey M. (1958) Acta Ophthalmol. 36, 837-844.
6. Fisher R.F. (1978) Interdiscipl. Topics Geront. 13, 131-142.
7. Wistow G.J. and Piatigorsky J. (1988) Annu. Rev. Biochem. 57, 479-504.
8. Piatigorsky J. and Wistow G. (1991) Science 252, 1078-1079.
9. Iwaki T., Kume-Iwaki A., Liem R.K. and Goldman J.E. (1989) Cell 57, 71-78.
10. Hackett E. and Thompson A. (1964) Lancet 2, 663-666.
11. Sandberg H.O. and Closs O. (1979) Scand. J. Immunol. 10, 549-554.
12. Angunawela I. (1987) Immunology 61, 363-368.
13. Nissen S.H., Andersen P. and Andersen H.M.K. (1981) Br. J. Ophthalmol. 65, 63-66.
14. Deschênes J., Baines M. and Anteka A. (1989) Invest. Ophthalmol. Vis. Sci. 30, 83, ARVO abstracts.
15. Luntz M.H. (1968) Exp. Eye Res. 7, 561-569.
16. Patel M., Shine B. and Murray P.I. (1990) Int. Ophthalmol. 14, 97-100.
17. Wirostko E. and Spalter H.F. (1967) Arch. Ophthalmol. 78, 1-7.
18. Laemmli U.K. (1970) Nature 227, 680-687.
19. O'Farell P.H. (1975) J. Biol. Chem. 250, 4007-4021.
20. de Jong W.W., Zweers A., Versteeg M. and Nuy-Terwindt E.C. (1984) Eur. J. Biochem. 141, 131-140.
21. Berbers G.A.M., Boerman O.C., Bloemendal H. and de Jong W.W. (1982) Eur. J. Biochem. 128, 495-502.
22. Towbin H., Staehelin T. and Gordon J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.
23. Mulders J.W.M., Hoekman W.A., Bloemendal H. and de Jong W.W. (1987) Exp. Cell Res. 171, 296-305.

24. Berbers G.A.M., Feenstra R.W., van den Bos R., Hoekman W.A., Bloemendal H. and de Jong W.W. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 7071-7020.
25. Berbers G.A.M., Hoekman W.A., Bloemendal H., de Jong W.W., Kleinschmidt T. and Braunitzer G. (1984) Eur. J. Biochem. 46, 467-479
26. Goldschmidt L., Goldbaum M., Walker S.M. and Weigle W.O. (1982) J. Immunol. 129, 1652-1657.
27. Ohga H, Katayama T, Egi K and Fujiwara H (1990) In: Usui M, Ohno S and Aoki K, (Eds) Ocular Immunology Today: Proceedings of the 5th international symposium on the immunology and immunopathology of the eye, Tokyo 13-15 March 1990 (pp 391-394) Elsevier Science Publishers, Amsterdam.

SUMMARY/SAMENVATTING

SUMMARY

To better understand how the eye lens refracts the incident light and how it is organized to yield a perfectly transparent tissue, it is of importance to know the structures of its composing elements. The major protein components of the eye lens are the crystallins, of which α-crystallin is the most abundant class in many vertebrates. α-Crystallin occurs as a large aggregate, composed of two types of subunits, αA- and αB-crystallin. The elucidation of the spatial conformation of α-crystallin becomes increasingly interesting considering the discoveries made during the last ten years. α-Crystallin was found to belong to the same protein family as the ubiquitous small heat shock proteins (HSPs), which are thought to play a crucial role in the survival of organisms under conditions of stress. Additionally, both subunits of α-crystallin occur in different cell types outside the lens. The expression of αB-crystallin is associated with various stress conditions and occurs at elevated levels in several diseased tissues. Moreover, it has recently been shown that α-crystallin can act as a molecular chaperone *in vitro*. The elucidation of the three-dimensional structure of α-crystallin might thus contribute to a better understanding of the function of the members of the α-crystallin/small HSP family.

It is likely that the common property of the α-crystallins and the small HSPs to form large aggregates is essential for their function. This thesis therefore focuses on the quaternary structure of α-crystallin. Additionally, it illustrates the structural and functional relationship between α-crystallin and mammalian small HSP.

In chapter 2 a study is described in which the mouse small HSP (HSP25) is compared with α-crystallin, with respect to several known properties of the latter, to extend the insight into their structural and functional similarities. One of the properties of α-crystallin is its ability to function as a molecular chaperone. Chaperones are proteins that prevent the formation of incorrect structures or interactions of other proteins and that unscramble any that do occur, as might result from heat shock or other forms of stress. It is shown that, like α-crystallin, HSP25 can function as a molecular chaperone, being able to prevent the heat-induced denaturation of βL-crystallin. HSP25 also is an efficient inhibitor of elastase and is found to be a substrate for tissue-type transglutaminase, like αB-crystallin. It is envisaged that α-crystallin and HSP25 might have similar structures, since they resemble each other in secondary structure, are equally stable towards dissociation by urea treatment and form viable mixed aggregates from any combination of subunits.

The α-crystallin and small HSP subunits are presumably composed of two globular domains and an extending C-terminal tail (chapter 1, ref. 30). It has been proposed that the C-terminal domain, which is the best conserved sequence within the α-crystallin/small HSP protein family, is the 'aggregation domain'. To test this hypothesis the N-terminal domain and C-terminal domain, including the flexible tail of αA-crystallin were isolated and tested for their ability to form aggregates. This study is described in chapter 3. It turned out that the C-terminal domain and tail (αA2Dt) forms dimers or tetramers, whereas the N-terminal domain exists in a less-defined high molecular weight multimeric form. To further explore the importance of the conserved C-terminal domain and tail, the corresponding protein fragments of αB-crystallin (αB2Dt) and HSP25 (HSP2Dt) were

isolated and investigated with regard to secondary and quaternary structure (Chapter 4). αA2Dt, αB2Dt and HSP2Dt have secondary structure elements that closely resemble those of the proteins from which they are derived, namely mainly β-pleated sheet structure and hardly any α-helical structure. This is in agreement with the proposal that the α-crystallin/small HSP subunits are composed of two domains with similar structural motifs. Besides, like their primary structures, their secondary structures are conserved. αB2Dt and HSP2Dt form large aggregates, which seems to be in contrast with the aggregation behaviour of αA2Dt. However, they are most probably composed of dimeric or tetrameric building blocks. The results were therefore taken to fit the rhombo-dodecahedric model for the quaternary structure of α-crystallin and the small HSPs (chapter 1, ref. 139).

In chapter 5, the effect of several point-mutations on the aggregation and chaperone activity of αA-crystallin is described. Evidence is found that certain hydrophobic residues are involved in intersubunit interactions. Since the mutated residues are located in both protein domains, the aggregation capacity cannot be ascribed simply to one protein domain. An evolutionarily well-conserved charged residue was found to be important for the structure and chaperone-activity of αA-crystallin.

Finally, anti-crystallin antibodies in the serum of patients with cataract were identified (Chapter 6). It turned out that the immune-system is tolerant for exactly those crystallins that occur extra-lenticularly (αA-, αB- and βB2-crystallin).

SAMENVATTING

De ooglens breekt het invallend licht en is geheel transparant. Om de werking van de ooglens beter te kunnen begrijpen is het van belang de structuur te kennen van de elementen waaruit de lens is opgebouwd. De belangrijkste eiwitcomponenten van de lens zijn de crystallines. In de lenzen van veel vertebraten komt α-crystalline in grote hoeveelheden voor. α-Crystalline vormt grote eiwitcomplexen, die zijn opgebouwd uit twee typen polypeptideketens, αA- en αB-crystalline. Het oplossen van de ruimtelijke structuur van α-crystalline wordt van steeds groter belang, gezien de ontwikkelingen van de laatste 10 jaar. Ontdekt werd dat α-crystalline tot dezelfde eiwitfamilie behoort als de kleine 'heat shock'-eiwitten (HSPs). Deze eiwitten worden verondersteld een belangrijke rol te spelen in het overlevingsproces van organismen, nadat ze aan stress zijn blootgesteld. Bovendien is ontdekt dat beide α-crystallineketens ook in diverse weefsels buiten de lens voorkomen. De expressie van αB-crystalline wordt verhoogd onder stress-condities. Bovendien is het nivo van αB-crystalline verhoogd bij degeneratieve afwijkingen van bepaalde organen. Tevens heeft men gevonden dat α-crystalline *in vitro* zich als een moleculaire chaperonne kan gedragen. Het ophelderen van de drie-dimensionale structuur van α-crystalline kan dus ook een bijdrage leveren aan een beter begrip van de functie van de leden van de eiwitfamilie van α-crystalline en de kleine HSPs. Het ligt voor de hand dat de opbouw van de aggregaten van α-crystalline en de kleine HSPs essentieel is voor hun functie. Dit proefschrift gaat daarom vooral in op deze 'quaternaire' structuur van α-crystalline. Daarnaast wordt de relatie tussen structuur en functie van α-crystalline en de kleine HSPs verduidelijkt.

In hoofdstuk 2 wordt een studie beschreven waarin eigenschappen van het kleine 'heat shock'-eiwit van de muis (HSP25) wordt vergeleken met die van α-crystalline, met het doel het inzicht in structurele en functionele overeenkomsten te vergroten. Eén van de eigenschappen van α-crystalline is het vermogen om zich als een moleculaire ch ·onne te gedragen. Dit zijn eiwitten die voorkómen dat andere eiwitten een verkeerde .tuur aannemen of die een eenmaal verkeerd gevouwen eiwit, dat bijvoorbeeld ontstaan is na een hitte-schok, ontwarren. Aangetoond werd dat ook HSP25 zich als een moleculaire chaperonne kan gedragen. HSP25 beschermt namelijk βL-crystalline tegen hitte-geïnduceerde denaturatie. Verder is aangetoond dat HSP25 een efficiënte inhibitor van elastase is en dat het een substraat is voor transglutaminase, evenals αB-crystalline. Aannemelijk werd gemaakt dat α-crystalline en de kleine HSPs vergelijkbare structuren hebben: de secundaire structuren lijken op elkaar, beide eiwitten zijn even stabiel wanneer ze met ureum worden gedissociëerd en elke combinatie van eiwitketens (αA, αB of HSP25) levert een aggregaat van de juiste grootte op.

De eiwitketens van α-crystalline en de kleine HSP-ketens zijn waarschijnlijk opgebouwd uit twee globulaire domeinen en een uitstekend C-terminaal staartje (hoofdstuk 1, ref. 30). Het C-terminale domein, dat de best geconserveerde sequentie van de eiwitfamilie is, wordt verondersteld het 'aggregatie-domein' te zijn. Om deze hypothese te testen werden het N-terminale domein en het C-terminale domein met staartje van αA-crystalline geïsoleerd en onderzocht op aggregatiegedrag. Deze studie is beschreven in hoofdstuk 3. Het bleek dat het C-terminale domein met staartje (αA2Dt) dimeren of tetrameren vormt.

Het N-terminale domein daarentegen heeft een moelijk definiëerbare multimere vorm. Om het belang van het geconserveerde C-terminale domein met staartje verder te onderzoeken zijn ook de overeenkomstige fragmenten van αB-crystalline (αB2Dt) en HSP25 (HSP2Dt) geïsoleerd. Vervolgens is de secundaire en quaternaire structuur van deze eiwitfragmenten onderzocht (Hoofdstuk 4). αA2Dt, αB2Dt en HSP2Dt hebben een secundaire structuur die sterk lijkt op de eiwitten waarvan ze zijn afgeleid. Ze bestaan voornamelijk uit 'β-pleated sheets' en bevatten weinig of geen α-helix. Dit stemt overeen met de veronderstelling dat de structuur van de α-crystalline en kleine HSP-polypeptideketens uit twee domeinen bestaat, met twee gelijkende structurele motieven. Daarnaast is de secundaire structuur van het C-terminale deel van de onderzochte leden van de eiwitfamilie goed geconserveerd, net zo als de primaire structuur. αB2Dt en HSP2Dt vormen grote aggregaten, hetgeen in tegenstelling lijkt te zijn met het aggregatiegedrag van αA2Dt. Hoogst waarschijnlijk echter zijn deze aggregaten opgebouwd uit dimere of tetramere eenheden. De resultaten worden beschouwd in overeenstemming te zijn met het rhombododecahedron model voor de quaternaire structuur van α-crystalline en de kleine HSPs (hoofdstuk 1, ref. 139).
In hoofdstuk 5 wordt het effect van enkele puntmutaties op het aggregatie- en chaperon-negedrag van αA-crystalline beschreven. Er zijn aanwijzingen gevonden dat bepaalde hydrofobe residuen betrokken zijn bij interacties tussen de polypeptideketens. Aangezien de gemuteerde residuen zich in beide eiwitdomeinen bevinden, kan het vermogen om aggregaten te vormen niet aan slechts één domein worden toegekend. Een geladen residu dat goed geconserveerd is gebleven tijdens de evolutie, bleek belangrijk te zijn voor de structuur en de chaperonne-activiteit van αA-crystalline.
Tenslotte werden antilichamen tegen crystallines in het serum van patienten met cataract geïdentificeerd (Hoofdstuk 6). Daaruit bleek dat het immuunsysteem tolerant is voor juist die crystallines die ook buiten de lens voorkomen (αA-, αB- en βB2-crystalline).

LIST OF PUBLICATIONS

Merck, K.B., de Haard-Hoekman, W.A., Oude Essink, B.B., Bloemendal, H. and de Jong, W.W. (1992) Expression and aggregation of recombinant αA-crystallin and its two domains. Biochim. Biophys. Acta 1130, 267-276.

Renkawek, K., de Jong, W.W., Merck, K.B., van Workum, F.P.A. and Bosman, G.J.C.G.M. (1992) αB-crystallin is present in reactive glia in Creutzfeldt-Jakob disease. Acta Neuropathol. 83, 324-327.

Merck, K.B., Groenen, P.J.T.A., Voorter, C.E.M., de Haard-Hoekman, W.A., Horwitz, J., Bloemendal, H. and de Jong, W.W. (1993) Structural and functional similarities of bovine α-crystallin and mouse small heat shock protein. J. Biol. Chem. 268, 1046-1052.

Merck,K.B., de Haard-Hoekman, W.A., Cruysberg, J.R.M., Bloemendal, H. and de Jong, W.W. (1993) Characterization of anti-crystallin autoantibodies in patients with cataract. Mol. Biol. Rep. (In Press).

Merck K.B., Horwitz, J., Kersten, M., Overkamp, P., Gaestel, M., Bloemendal, H. and de Jong, W.W. Comparison of the homologous carboxy-terminal domain and tail of α-crystallin and small heat shock protein. (Submitted).

Merck, K.B., Aendekerk, J., Horwitz, J., Takemoto, L., Slingsby, C., Bloemendal, H. and de Jong, W.W. Exploring the aggregation and chaperone behavior of αA-crystallin by site-directed mutagenesis. (Submitted).

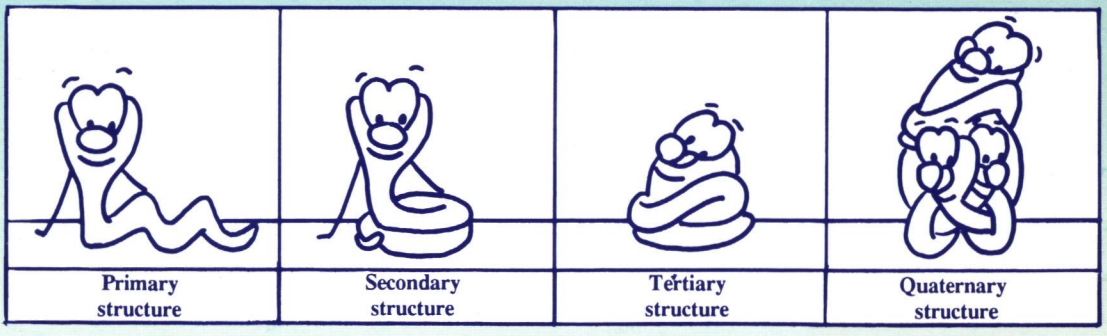
CURRICULUM VITAE

Karin Merck werd geboren op 28 maart 1964 te Dordrecht. In juni 1982 behaalde zij het VWO-diploma aan het Strabrecht College te Geldrop. Zij besloot vervolgens de goede gewoonte van een ver familielid na te volgen en begon in september 1982 de studie Scheikundige Technologie aan de Technische Universiteit van Eindhoven. Zij deed haar afstudeerstage bij de vakgroep Organische Chemie. In juni 1987 behaalde zij het ingenieursdiploma (met lof). Van september 1987 tot maart 1992 werkte zij als onderzoeker in opleiding aan de afdeling Biochemie van de Katholieke Universiteit te Nijmegen. Onder leiding van Prof. Dr. W.W. de Jong en Prof. Dr. H. Bloemendal werd het onderzoek verricht dat in dit proefschrift is beschreven. Tijdens haar o.i.o.-schap behaalde zij het diploma 'Deskundigheid Stralingshygiëne, nivo C' en leverde zij een bijdrage aan het practisch biochemie-onderwijs van scheikundestudenten. Zij bezocht diverse congressen: Spetsai Summerschool on Molecular and Cellular Biology: "Protein and Genetic Engineering (Griekenland, 1988), het 9e en 10e International Congress on Eye Research (Helsinki, 1990 en Stresa, 1992) en het Keystone (UCLA) Symposium on 'Protein Folding, Structure and Function' (Keystone, 1991). Sinds 1 februari 1993 werkt zij als post-doc bij het instituut voor Biomembranen aan de Universiteit van Utrecht.

Dit proefschrift werd mede mogelijk gemaakt door

FILTRON Technology B.V.

Filtron Technology B.V.
Hoge Hil 28
4822 AD Breda
Telefoon 076-421777
Telefax 076-423340


Primary structure
Secondary structure
Tertiary structure
Quaternary structure